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NEW EXPERIMENTS ON ANOMALOUS OSMOSIS

By KARL SOLLNER and IRVING ABRAMS

(*From the Department of Physiology, University of Minnesota, Minneapolis*).

(Received for publication, May 1, 1940)

For several years we have been aware of the fact that the well known experiments of Jacques Loeb,[1] Bartell,[2] and others[3] concerning anomalous osmosis[4] through collodion membranes could not be reproduced satisfactorily. The effects obtained were usually insignificant or poor at best.

In connection with new work relating to the general theory[5] of anomalous osmosis it became necessary to clear up this matter, especially since it seemed that any clarification might cast considerable light on some basic membrane properties.

When an electrolyte diffuses through a membrane from a more concentrated to a more dilute solution, the sign and extent of anomalous osmosis are correlated rather clearly[6] with the electrokinetic properties (ζ-potential) of the membrane and with the dynamic membrane potential (ϵ-potential) that is observed. Both of these quantities depend ultimately on the electrical structure of the solid-liquid interphase in the pores of the membrane.

The obvious starting point for any experimental investigation was to test different brands of collodion. The following preparations were tested: Parlodion Mallinckrodt, 5 per cent dissolved in 75 per cent alcohol, 25 per

[1] Loeb, J., *J. Gen. Physiol.*, 1918–19, **1,** 717; 1919–20, **2,** 173, 255, 387, 563, 577, 659, 673; and many other papers in the succeeding volumes of the same Journal.

[2] Bartell, F. E., and Madison, O. E., *J. Physic. Chem.*, 1920, **24,** 444; Bartell, F. E., and Carpenter, D. C., *J. Physic. Chem.*, 1923, **27,** 101, 252, 346; Bartell, F. E., Membrane potentials and their relation to anomalous osmosis, in Mathews, J. H., Colloid symposium monograph, Department of Chemistry, University of Wisconsin, Madison, 1923, **1,** 120; and many other publications.

[3] Preuner, G., and Roder, O., *Z. Elektrochem.*, 1923, **29,** 54; Girard, P., *Compt. rend. Acad. sc.*, 1908, **146,** 927.

[4] By anomalous osmosis we mean those phenomena of liquid mass movement which occur when electrolyte solutions dialyze through membranes but are unexplainable on the basis of the laws of normal osmosis.

[5] Sollner, K., *Z. Elektrochem.*, 1930, **36,** 36, 234; Sollner, K., and Grollman, A., *Z. Elektrochem.*, 1932, **38,** 274; *Tr. Electrochem. Soc.*, 1932, **61,** 477, 487; Sollner, K., *Kolloid-Z.*, 1933, **62,** 31.

cent ether; Collodion Merck, U.S.P.X.; Collodion Baker, U.S.P.; Collodium Schering-Kahlbaum "pro analysi;" Collodium Schering-Kahlbaum "für Membranen;" Collodium Schering-Kahlbaum D.A.B.6.

When tested with uni-univalent electrolytes, *e.g.* KCl, in which we were particularly interested, the first four of these brands of collodion gave no significant positive results. Schering-Kahlbaum collodion "für Membranen" gave doubtful effects. Only Schering-Kahlbaum D.A.B.6. collodion gave decidedly positive results of the order of magnitude described by Loeb.[6] Correspondingly, bi- and trivalent ions gave only very moderate results with membranes of the first four named brands of collodion. Schering-Kahlbaum "für Membranen" gave somewhat higher values. Here too, only membranes prepared from collodium Schering-Kahlbaum D.A.B.6. yielded results approaching in magnitude those reported by Loeb.[6]

This particular brand of collodion, incidentally, was used by Loeb in his experiments nearly twenty years ago. Michaelis[7] likewise in his classical experiments on the dried collodion membrane used Schering-Kahlbaum D.A.B.6, as this brand also gave in his hands the most characteristic and reproducible results. No satisfactory explanation of the cause of this different behavior of the several brands of collodion has been noted in the literature. Thus additional insight into the ultimate cause of the peculiar behavior of Schering-Kahlbaum D.A.B.6 collodion promised to cast additional light on the results of Loeb and Michaelis.

On the basis of observations to be reported in detail later, it was concluded that certain impurities or groups foreign to the pure ideal nitrocellulose were responsible for the "activity" of the actual collodion. The purer brands show less activity. The high efficiency of Schering-Kahlbaum D.A.B.6 collodion is due to its high content of groups foreign to pure nitrocellulose. More specifically, it was concluded that COOH groups, due to the presence of pectic substances or to oxidation, cause the relatively

[6] Experiments carried out about eight years ago with this brand of collodion gave decidedly less positive results than our present ones carried out with recently acquired Schering-Kahlbaum D.A.B. 6 collodion.

[7] Michaelis, L., and Fujita, A., *Biochem. Z.*, Berlin, 1925, **158**, 28; 1925, **161**, 47; 1925, **164**, 23; Michaelis, L., and Dokan, S., *Biochem. Z.*, Berlin, 1925, **162**, 258; Michaelis, L., and Hayashi, K., *Biochem. Z.*, Berlin, 1926, **173**, 411; Michaelis, L., and Perlzweig, W. A., *J. Gen. Physiol.*, 1926–27, **10**, 575; Michaelis, L., McEllsworth, R., and Weech, A. A., *J. Gen. Physiol.*, 1926–27, **10**, 671; Michaelis, L., Weech, A. A., and Yamatori, A., *J. Gen. Physiol.*, 1926–27, **10**, 685; Michaelis, L., *Bull. Nat. Research Council, No. 69*, 1929, 119; *Kolloid-Z.*, 1933, **62**, 2, and other publications.

great activity characteristic of membranes prepared from this particular brand of collodion.

This conclusion immediately suggested a method of increasing the activity of collodion membranes by oxidation. Following a suggestion of Meyer and Sievers,[8] the membranes were oxidized for several hours with NaOBr solution, prepared by saturating normal NaOH with molecular bromine. The activity of the membranes increases with increasing oxidation time. The better brands of collodion withstand 24 hours oxidation without damage, whereas the poorer grades leak and cannot be oxidized so long. We have not yet made a study of the optimum conditions for maximum activity.

The experimental technique is as follows: Collodion bags are cast in 30 × 110 mm. test tubes and allowed to dry several minutes, the suitable drying time varying considerably with different brands of collodion. Next the bags are filled with water; they loosen from the glass spontaneously and are tied to glass rings with thread while still filled with water. Following this, they are kept in covered glass containers under water to which thymol has been added as a preservative. The membranes so prepared are fitted to rubber stoppers provided with a long capillary tubing (inner diameter about 1½ mm.). Following the suggestion of Loeb,[9] membranes were selected which, when filled with ¼ molar sugar solution and placed in water for 20 minutes, yield an osmotic rise of about 120 mm. of liquid in the capillary manometer. The adjustment of the zero reading is facilitated by a small glass syphon provided with a stopcock, allowing the rapid adjustment of the meniscus in the manometer to the proper level corresponding to the capillary rise over the outside solution.

The bag chosen for actual use is filled with solutions of varying (in our experiments decreasing) concentrations of first sugar, then KCl, K_2SO_4, and K_3-citrate. The rise of the meniscus in the manometer is noted 20 minutes after the bag is placed in water.

In the accompanying figures, the abscissae show molar concentrations in the collodion bag on a logarithmic scale, the ordinates the rise in level of the liquid in the manometer after 20 minutes (with the exception of the values for Loeb's K_3-citrate curve (Fig. 1) which are given after 10 minutes).

Fig. 1 gives for comparison a characteristic set of curves published by Loeb.[10]

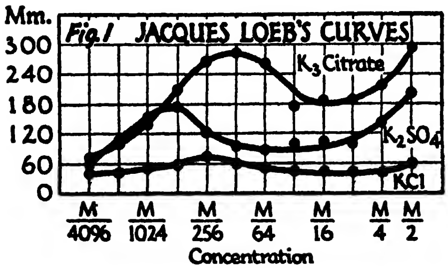
Fig. 2 shows the behavior of a membrane cast from one of the less active brands of collodion (Merck U.S.P.). This behavior is very characteristic and membranes cast from one particular lot of collodion under the same conditions give astonishingly reproducible results when properly selected

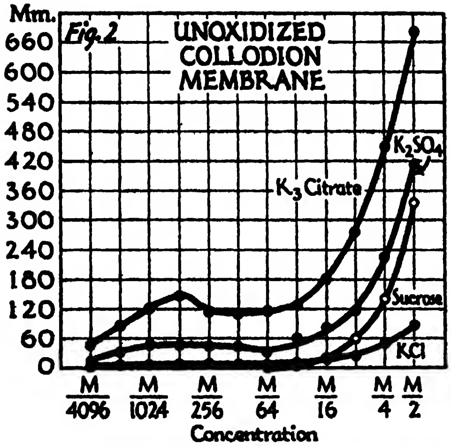
[8] Meyer, K. H., and Sievers, J.-F., *Helv. Chim. Acta*, 1936, **19**, 665.

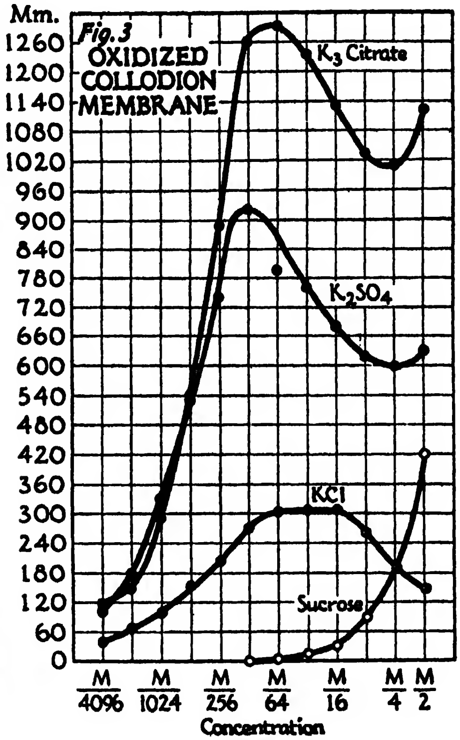
[9] Loeb, J., *J. Gen. Physiol.*, 1919–20, **2**, 93.

[10] Loeb, J., *J. Gen. Physiol.*, 1919–20, **2**, 564.

after the test with sugar (120 mm. rise after 20 minutes). The curves given in Fig. 2 were actually obtained with two membranes, as the first one was damaged in the middle of the K_3-citrate curve. Another one was substituted giving the same sugar value and satisfactory agreement within a few millimeters at several test points of the KCl, K_2SO_4, and the unfinished K_3-citrate curves.







This latter membrane was then oxidized for 24 hours, washed thoroughly, and again tested with sugar, KCl, K_2SO_4, and K_3-citrate in the order indicated. The values so obtained with the "activated" membrane are given in Fig. 3.

The difference between the unoxidized and the oxidized membrane is quite striking. The activity of the oxidized membrane surpasses by far the activity of those used by Loeb.

The differences would be still larger if one measured volumes transferred against a zero pressure and not pressure rises with a progressively increasing back filtration.

The oxidation method affords a simple and rational means of "activating"

membranes of otherwise inactive collodion in order to reproduce the Loeb experiments on anomalous osmosis. In addition it seems to open up a rather promising field of membrane research, for example, the comparison of membranes with nearly equal permeability for non-electrolytes but showing remarkable differences in their behavior towards electrolytes. The cause for this difference obviously has to be looked for in the great difference of the charge densities in the pores of the unoxidized and the oxidized membranes.

SUMMARY

1. It is impossible to reproduce Loeb's observations on anomalous osmosis with membranes prepared from relatively pure brands of collodion, whereas positive effects can be obtained using collodion containing acidic impurities.

2. The inactive (purer) collodion membranes may be activated by oxidation with NaOBr solution.

3. Properly oxidized membranes give much greater anomalous osmotic effects than those described by Loeb.

CHEMICAL RESTORATION IN NITELLA

IV. Effects of Guanidine

By W. J. V. OSTERHOUT

(*From the Laboratories of The Rockefeller Institute for Medical Research*)

(Received for publication, May 20, 1940)

The irritability[1] of *Nitella* and likewise its ability to distinguish electrically between Na^+ and K^+ (potassium effect) may be removed by leaching with distilled water. This apparently dissolves out a group of organic substances called for convenience *R*.[2]

Since presumably very little *R* is present it is not probable that enough can be obtained for analysis. We may, however, try to learn something about its nature by seeking substitutes which resemble it in being able to restore irritability and the potassium effect.[2]

Substances of this sort occur in blood.[3] To a certain extent ammonia and some of its compounds can take the place of *R*.[4] Guanidine[5] has a similar action and some of its effects will be briefly described in this paper.

Cells were kept in distilled water[6] until they had lost their irritability and the potassium effect.[7] They were then soaked in guanidine hydrochloride[8] for various lengths of time and tested for irritability and the

[1] By this is meant the ability to produce propagated action currents.

[2] Osterhout, W. J. V., *J. Gen. Physiol.*, 1939–40, **23**, 429.

[3] Osterhout, W. J. V., *J. Gen. Physiol.*, 1935–36, **19**, 423.

[4] Osterhout, W. J. V., *J. Gen. Physiol.*, 1934–35, **18**, 987.

[5] Guanidine, $NH{:}C(NH_2)_2$, is a strong base.

[6] The cells, after being freed from neighboring cells, stood in the laboratory at 15° ± 1°C. in Solution A (*cf.* Osterhout, W. J. V., and Hill, S. E., *J. Gen. Physiol.*, 1933–34, **17**, 87) for several days. They belonged to Lot B (*cf.* Hill, S. E., and Osterhout, W. J. V., *Proc. Nat. Acad. Sc.*, 1938, **24**, 312).

The measurements were made on *Nitella flexilis*, Ag., using the technique described in former papers (Hill, S. E., and Osterhout, W. J. V., *J. Gen. Physiol.*, 1937–38, **21**, 541). Temperature about 20–26°C.

There was no indication of injury in these experiments.

[7] During the leaching in many cases the potassium effect disappears before the irritability and the treatment with guanidine may restore the irritability earlier than the potassium effect.

[8] $NHC(NH_2)_2 \cdot HCl$.

potassium effect. In many cases both of these were restored by the treatment with guanidine. In some cases the irritability was restored after 15 seconds in 0.1 M guanidine hydrochloride. In other cases a much longer treatment was needed, especially when the reagent was applied at lower concentrations (0.01 to 0.0001 M). Much apparently depends on the condition of the cells[9] which appear to be highly variable in this respect: this is also true of the restoration of the potassium effect.[10]

During the treatment single peaks persisted for a time but after the potassium effect was restored double peaks made their appearance, as might be expected in view of what has been stated in previous papers.[11]

We do not know whether guanidine acts like *R* or is a constituent or catalyst of reactions forming *R*. But the rapidity with which guanidine produces its effects in some cases suggests that it does not merely cause *R* to come out of the vacuole, as has been suggested in connection with the restorative action of certain inorganic salts.[12]

It should be noted that the rapid restoration of irritability presumably involves penetration of guanidine to the inner protoplasmic surface abutting on the vacuole. The thickness of the protoplasm is less than 10 microns and may in some places be considerably less, and the penetration of an organic substance, such as guanidine, may be rapid.

The fact that double-peaked action curves make their appearance when the potassium effect has been restored and the outer protoplasmic surface has become sensitive to K^+ supports the suggestion made in previous papers.[11] According to this the double peaks depend upon the outward movement of K^+ which sets up a positive P.D. when it reaches the outer protoplasmic surface provided the latter is sensitive to K^+. When it is not sensitive, as in leached cells, we see only a single peak but when it has been made sensitive to K^+ by treatment with guanidine double-peaked action curves make their appearance.

SUMMARY

Leaching in distilled water may remove irritability and the potassium effect in *Nitella* but both of these may be restored by appropriate treatment with guanidine.

[9] Some cells did not respond readily to treatment with guanidine.

[10] When the guanidine has not acted sufficiently the potassium effect may be delayed (Hill, S. E., and Osterhout, W. J. V., *J. Gen. Physiol.*, 1938–39, **22**, 107) or incomplete.

[11] Osterhout, W. J. V., and Hill, S. E., *J. Gen. Physiol.*, 1939–40, **23**, 743.

[12] Osterhout, W. J. V., and Hill, S. E., *Proc. Nat. Acad. Sc.*, 1939, **25**, 3.

THE EXPERIMENTAL PRODUCTION OF DOUBLE PEAKS IN CHARA ACTION CURVES AND THEIR RELATION TO THE MOVEMENT OF POTASSIUM

BY W. J. V. OSTERHOUT AND S. E. HILL

(*From the Laboratories of The Rockefeller Institute for Medical Research*)

(Received for publication, May 20, 1940)

Chara offers an interesting contrast to *Nitella*: in the latter the action curve has two peaks but in *Chara* there is only one.

In *Nitella* the first peak has been accounted for on the ground[1] that when K^+, moving outward[2] from the sap, reaches the outer protoplasmic surface it sets up a positive P.D. which makes the action curve fall as soon as the first rise (the spike) is completed.[1] This fall in the curve occurs only when the outer protoplasmic surface is sensitive to K^+. When it is not sensitive to K^+ we find no change of P.D. when 0.01 M NaCl in external contact with the cell is replaced by 0.01 M KCl (*i.e.* no "potassium effect"). We may therefore expect only one peak in the action curve. This situation exists in *Nitella* when the outer protoplasmic surface is made insensitive to K^+ by treatment with distilled water.[3]

In *Chara* the outer protoplasmic surface[4] is normally insensitive to K^+, *i.e.* shows no potassium effect, and the action curve has only one peak, as would be anticipated. This is illustrated[5] in Fig. 1.

If the outer surface could be made sensitive to K^+ we might expect two peaks. This expectation is realized when the outer surface is made sensi-

[1] Osterhout, W. J. V., *J. Gen. Physiol.*, 1934-35, **18,** 215.

[2] The effect of K in *Nitella* and in *Chara* predominates to such an extent that it alone is mentioned in the following discussion.

[3] Osterhout, W. J. V., and Hill, S. E., *J. Gen. Physiol.*, 1939-40, **23,** 743.

[4] Presumably the inner protoplasmic surface is sensitive to K^+ as in *Nitella* and the outwardly directed P.D. of about 100 mv. usually present when the cell is in pond water is presumably due to the concentration gradient of K^+ across the inner protoplasmic surface.

[5] The cells, after being freed from neighboring cells, stood in the laboratory at 15° ± 1°C. in Solution A (*cf.* Osterhout, W. J. V., and Hill, S. E., *J. Gen. Physiol.*, 1933-34, **17,** 87).

The experiments were performed on *Chara coronata*, Ziz. (this is an uncorticated *Chara* with large naked cells like those of *Nitella*, completely accessible to reagents).

There was no indication of injury in these experiments.

tive to K^+ by guanidine which has been used to produce double peaks in leached cells of *Nitella*.[6]

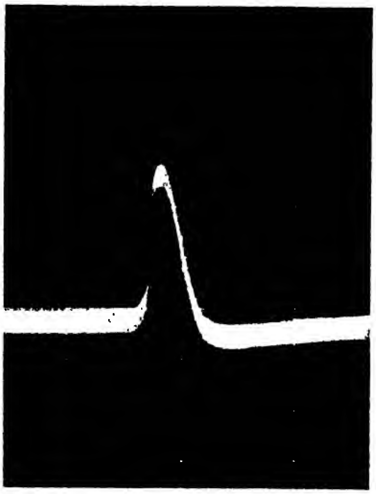


FIG. 1. Action curve in a normal cell of *Chara* which shows no potassium effect; *i.e.*, the outer protoplasmic surface is not sensitive to K^+.

The spot recorded, *D*, was in contact with 0.001 M KCl: it was connected through the recording galvanometer with a spot *F* killed by chloroform and having in consequence a P.D. of zero. Hence the action curve is monophasic.

The cell was freed from neighboring cells and kept for 2 days in Solution A at 15 ± 1°C. The record was made at 24°C. Vertical marks 5 seconds apart.

The treatment consists in soaking the cells for various periods in 0.1 to 0.001 M guanidine hydrochloride and then testing[7] them for the potassium effect and for irritability.[8]

In many cases this treatment produces the potassium effect but the time required is very variable. In some cases less than 4 minutes in 0.01 M guanidine hydrochloride[9] sufficed: in other cases a much longer exposure was necessary, especially at lower concentrations (0.01 to 0.001 M). The potassium effect is usually less than in *Nitella*.

Prior to the application of guanidine we find only rounded single peaks (as seen in Fig. 1) and no potassium effect. Guanidine cannot only bring about a potassium effect but, along with this, double peaks appear, such as are seen[10] in Figs. 2 to 4.

During this process single peaks may persist for a longer or shorter time at certain places.[11] This may be due to the fact that the potassium effect is not fully developed at these places. It appears to depend on a group of organic substances, called for convenience *R*. When but little *R* is present in the outer protoplasmic surface the outwardly moving K^+

[6] Osterhout, W. J. V., *J. Gen. Physiol.*, 1940 41, **24,** 7.

[7] The cells were tested for potassium effect and irritability before the treatment. The potassium effect was always absent: as a rule irritability was present.

[8] By this is meant the ability to give propagated action currents when stimulated electrically.

[9] $NHC(NH_2)_2 \cdot HCl$.

[10] Curves of this form are found in *Nitella* both in untreated cells and in leached cells treated with various restorative agents.

[11] There may even be some fluctuation in the form of the curve at the same spot.

may not produce much positive potential and thus there will be no sudden change in the curve. Moreover the guanidine, penetrating into the aqueous layer of the protoplasm, may tend to act like NaCl in favoring single peaks,[12] or may promote protoplasmic motion and thus make the moving boundary of K^+ less sharp, as discussed in a previous paper:[3] this would tend to promote single peaks.

FIG. 2. Action curve of a cell of *Chara* treated with 0.01 M guanidine hydrochloride for 14 minutes (the potassium effect was produced by this treatment: hence the outer surface was sensitive to K^+).

The curve falls below the original level and then rises at the end: this is often found in *Chara*.

The spot recorded, *D*, was in contact with 0.01 M guanidine hydrochloride and was connected through the galvanometer to a spot *G* in contact with 0.05 M KCl which kept the P.D. constant approximately at zero. In consequence the action curve is monophasic.

The cell was freed from neighboring cells and kept for 21 days in Solution A at 15 ± 1°C. The record was made at 23°C. Vertical marks 5 seconds apart.

The experiments indicate that the movement of potassium is as important in determining the shape of the action curve as in *Nitella*.[1, 3] The outwardly directed[13] (positive[14]) P.D. normally present in *Chara* is presumably due chiefly to the outwardly directed concentration gradient of K^+ across the inner protoplasmic surface. When an action current occurs this surface presumably becomes more permeable. The concentration gradient then disappears and with it the positive P.D. This causes the rise in the action curve (spike). Then K^+ is carried back into the sap by the forces which normally cause such movement[15] in the resting state of the cell. This produces recovery[16] and the curve falls.

When the outer protoplasmic surface has been made sensitive to K^+ by treatment with guanidine we observe an additional feature, *i.e.* on reaching the outer protoplasmic surface K^+ sets up more or less positive

[12] Hill, S. E., and Osterhout, W. J. V., *J. Gen. Physiol.*, 1938 39, **22**, 91.

[13] This is about 100 mv. when the cell is in contact with pond water.

[14] The P.D. is called positive when the positive current tends to flow from the sap across the protoplasm to the external solution.

[15] Osterhout, W. J. V., *J. Gen. Physiol.*, 1932-33, **16**, 157.

[16] This is usually more rapid in *Chara* than in *Nitella*.

P.D. This may be sufficient to cause a drop in the curve, as in Figs. 2 to 4. Or it may merely halt the course of recovery and so delay the fall of the curve.

As K^+ penetrates the outer protoplasmic surface and its concentration gradient across this surface decreases the positive P.D. will fall off.

FIG. 3

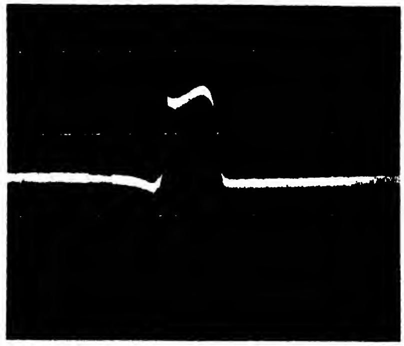


FIG. 4

FIG. 3. Action curve of a cell of *Chara* treated with 0.01 M guanidine hydrochloride for 7 minutes and 46 seconds (the potassium effect was produced by this treatment: hence the outer surface was sensitive to K^+).

The spot recorded, *E*, was in contact with 0.01 M guanidine hydrochloride and was connected through the galvanometer to a spot *G* in contact with 0.01 M KCl which kept the P.D. constant approximately at zero. In consequence the action curve is monophasic.

The cell was freed from neighboring cells and kept for 11 days in Solution A at 15 ± 1°C. The record was made at 23°C. Vertical marks 5 seconds apart.

FIG. 4. Action curve of a cell of *Chara* treated with 0.01 M guanidine hydrochloride for 7 minutes and 46 seconds (the potassium effect was produced by this treatment: hence the outer surface was sensitive to K^+).

The spot recorded, *E*, was in contact with 0.01 M guanidine hydrochloride and was connected through the galvanometer to a spot *G* in contact with 0.01 M KCl which kept the P.D. constant approximately at zero. In consequence the action curve is monophasic.

The cell was freed from neighboring cells and kept for 11 days in Solution A at 15 ± 1°C. The record was made at 23°C. Vertical marks 5 seconds apart.

As the magnitude of this positive P.D. will depend chiefly on the concentration gradient of K^+ across the outer protoplasmic surface it will be affected by a variety of factors. The greatest concentration gradient will be found when K^+ travels outward from the sap in the form of a moving boundary.[3] Anything which tends to disturb this, such as protoplasmic motion, will lessen the concentration gradient across the outer protoplasmic surface and consequently the positive P.D.

The higher the concentration of K^+ in the aqueous layer of the protoplasm before stimulation the less will be the effect on the P.D. of the outwardly moving K^+.

The P.D. will also depend on the sensitivity of the outer surface to K^+ and this in turn will depend on a variety of factors. It is not known how the guanidine acts to sensitize the surface. It might act like certain organic substances (*R*) responsible for the potassium effect. Or it might be a constituent or a catalyst of the reactions by which such substances are formed. Their concentration in the outer protoplasmic surface might fluctuate as the result of diffusion and chemical change.

SUMMARY

The action curve in *Chara* seems to depend (as in *Nitella*) on the outward movement of K^+ from the sap.

Presumably the increase in permeability in the inner protoplasmic surface and the outward movement of K^+ destroy the concentration gradient of K^+ across the inner protoplasmic surface. Hence the outwardly directed P.D. disappears, causing the rise (spike) of the action curve.

The outer protoplasmic surface is normally insensitive to K^+. But when it is made sensitive to K^+ by treatment with guanidine the outwardly moving K^+ sets up a positive P.D. on reaching the outer surface and this causes the action curve to fall, producing a peak. Then the curve has 2 peaks, the second being due to the process of recovery.

The action curve thus comes to resemble that of *Nitella* in which the outer protoplasmic surface is normally sensitive to K^+.

CRYSTALLINE RIBONUCLEASE

BY M. KUNITZ

(*From the Laboratories of The Rockefeller Institute for Medical Research, Princeton, New Jersey*)

(Received for publication, June 3, 1940)

The presence in pancreas of a heat stable enzyme capable of digesting yeast nucleic acid was described by W. Jones in 1920 (1). He found that the digestion was not accompanied by any liberation of free phosphoric acid. Jones' observations were recently confirmed by Dubos (2). Dubos and Thompson (3) carried out a partial purification of the enzyme by means of acetone, and named the enzyme "ribonuclease." Schmidt and Levene (4) considered the name "ribonucleodepolymerase" to be more appropriate for the enzyme since they considered that the enzyme had only a depolymerizing effect on yeast nucleic acid without the production of mononucleotides.

This paper describes the method of preparation as well as some of the properties of a crystalline protein recently isolated by the writer (5) from beef pancreas which acts as a powerful digestive enzyme on yeast nucleic acid. The enzymatic activity of the isolated crystalline protein appears to correspond to the nuclease activity described by the authors mentioned before. The name "ribonuclease" has been provisionally retained for the new crystalline enzyme until definite information becomes available concerning the chemical structure of the split products of digestion of yeast nucleic acid by this enzyme.

Crystalline ribonuclease is a soluble protein of albumin type. Its molecular weight is about 15,000. It contains very little, if any, phosphorus. It yields on hydrolysis tyrosine but not tryptophane. Crystalline ribonuclease is very stable over a wide range of pH. The activity is only very slowly diminished irreversibly when the protein is heated at 100°C. at pH 2.0. Heating at pH 5.0 or higher brings about a gradual denaturation of the protein with a corresponding percentage loss of enzymatic activity.

The digestion of yeast nucleic acid by ribonuclease is accompanied by a gradual formation of free acid groups without any significant liberation of free phosphoric acid. The split products, unlike the undigested yeast

nucleic acid, are not precipitable by glacial acetic acid or by 0.5 M hydrochloric acid. The products of digestion readily diffuse through collodion or cellophane membranes that are impermeable to the undigested yeast nucleic acid. Crystalline ribonuclease does not appear to exert any significant digestive action on thymus nucleic acid.

Dr. R. J. Dubos kindly tested the effect of the new crystalline material on the staining characteristics of two strains of pneumococcus (heat killed). He found "that their staining characteristics are altered after a few hours incubation;" like the material which he described, the new crystalline protein "decreases the affinity of the bacterial cells for basic dyes" (personal communication from Dr. Dubos).

EXPERIMENTAL

I. *Method of Isolation of Crystalline Ribonuclease*

The method of isolation consists essentially in separating the proteins of an acid extract of fresh beef pancreas by means of fractional precipitation with ammonium sulfate. The bulk of the ribonuclease protein is found in that fraction which is soluble in 0.6 saturated ammonium sulfate and insoluble in 0.8 saturated ammonium sulfate solution. The details of the method are as follows:

1. Preliminary Treatment.—Beef pancreas (about 20 pounds) is removed from the animals immediately after slaughter and immersed at once in enough ice cold 0.25 N sulfuric acid to cover the glands. It can then be stored at 5°C. for a day or so, or worked up immediately. The pancreas is removed from the acid, cleaned of fat and connective tissue, and then minced in a meat chopper. The minced pancreas is suspended in an equal volume of cold 0.25 N sulfuric acid and is stored at about 5°C. for 18–24 hours. It is then strained through cheese cloth. The strained fluid is brought to 0.6 saturation of ammonium sulfate by dissolving 390 gm. of salt in each liter of strained fluid. The mixture is filtered through 50 cm. fluted filter paper (No. 612 Eaton and Dikeman Co., Mt. Holly Springs, Pa., or No. 1450½ Schleicher and Schüll). The clear filtrate (0.6 F) is used for the preparation of ribonuclease while the residue on the paper (0.6 P) can be used for the isolation of chymotrypsinogen, trypsinogen, trypsin, and trypsin inhibitor compound.[1]

The clear filtrate (0.6 F) is brought with solid ammonium sulfate to 0.8 saturation (140 gm. per liter of filtrate) and the precipitate formed is allowed to settle for 2 days in the cold room. The settling is greatly facilitated by occasional stirring and removal of foam during the first day of standing. The clear supernatant fluid is siphoned off and rejected, while the remaining suspension is filtered with suction through hardened paper; yield about 30 gm.

2. Isolation of Ribonuclease Crystals.—Each 10 gm. of the semi-dry precipitate is

[1] The precipitate (0.6 P) is scraped off the filter paper and suspended in about 3 volumes of water. The procedure for further treatment is the same as described by Kunitz and Northrop for the treatment of the original acid extract of fresh beef pancreas in the preparation of chymotrypsinogen etc. (6).

dissolved in 50 ml. distilled water, the pH of the solution is adjusted by means of a few drops of 5 N sodium hydroxide to pH 4.8, and then 50 ml. of saturated ammonium sulfate is added with stirring.[2] The solution is filtered with suction through soft paper with the aid of about 1 gm. of Filter-Cel.[3] The clear filtrate is brought to pH 4.2 (tested with methyl orange) by means of a few drops of 1 N sulfuric acid and then 66 ml. saturated ammonium sulfate is added per 100 ml. of filtrate. The saturated ammonium sulfate is added slowly with stirring. The precipitate formed is filtered with suction through hardened paper; yield about 8 gm. Each 10 gm. of final filter cake is dissolved

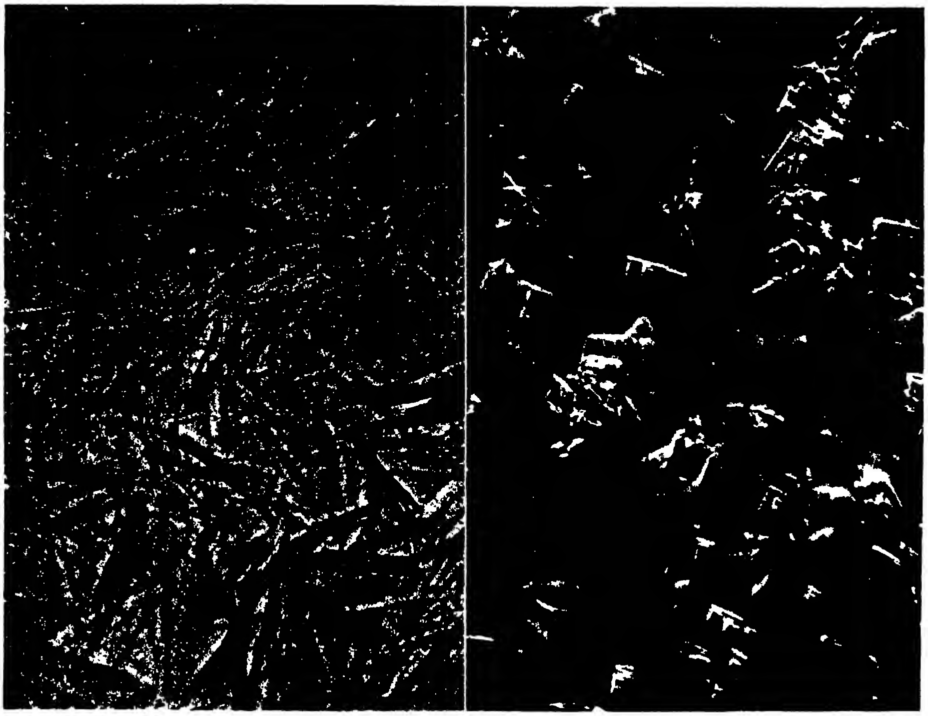


FIG. 1. Crystals of ribonuclease. × 248 and 190

in 10 ml. of water and is refiltered with suction through soft paper on a small Büchner funnel with the aid of 0.5 gm. Filter-Cel. The residue is washed several times with 2–3 ml. water. The combined filtrate and washings are made up with water to a volume of 20 ml. and 7 ml. saturated ammonium sulfate is added with stirring. The clear solution is left at 20–25°C. Crystals of ribonuclease in the form of thin, long plates or fine needles gradually appear (Fig. 1). The crystals are filtered after 2 or 3 days; yield

[2] The pH is determined approximately by mixing on a test plate 1 drop of 0.01 per cent neutralized methyl red (or any other indicator depending on the pH range) with 1 drop of the solution and the color is compared with the color of 1 drop standard buffer solution mixed with the indicator on the plate.

[3] Supplied by Johns-Manville Corporation, New York.

1–2 gm. More saturated ammonium sulfate is added to the filtrate until a slight turbidity is formed. A second crop of crystals appears after several days; yield 2–4 gm.

3. Alternate Method of Isolation of Ribonuclease Crystals.—The following method, described in the preliminary publication, is somewhat simpler in operation but it yields fewer crystals. The 0.25 N acid extract of the minced pancreas is brought to 0.7 saturation with solid ammonium sulfate and filtered. The filtrate is then brought to 0.8 saturation with more ammonium sulfate and is refiltered with suction.

Crystallization.—10 gm. of the semi-dry precipitate is dissolved in about 10 ml. of water. The solution is filtered with the aid of about 0.5 gm. of Filter-Cel through soft filter paper on a small Büchner funnel; the residue on the paper is washed with water. The combined filtrate and washings are brought to a final volume of 20 ml. Saturated ammonium sulfate is then added slowly with stirring until a very faint turbidity appears. The pH of the solution is adjusted first to about pH 5.0 with the aid of a few drops of 1.0 N sodium hydroxide and then to pH 4.2 by means of 1.0 N sulfuric acid. The solution is allowed to stand at about 20°C. An amorphous precipitate rapidly forms. This changes within 1 or 2 days into a mass of fine needles or aggregates of long thin plates. The crystals are filtered after 2 or 3 days. The filtrate on further addition of saturated ammonium sulfate yields more crystals.

4. Recrystallization.—Each 10 gm. of semi-dry filter cake of crystals is dissolved in 20 ml. of water. This solution is filtered with suction through soft paper with the aid of 1 gm. of Filter-Cel. The residue is washed with water. The combined filtrate and washings are made up to 30 ml. with water. 10 ml. saturated ammonium sulfate is added. Rapid crystallization takes place at 20–25°C. The crystals are filtered off after 1 or 2 days; yield about 5 gm. The filtrate on further addition of saturated ammonium sulfate gives more crystals; yield about 2 gm.

5. Recrystallization in Alcohol.—Ribonuclease is readily recrystallizable in dilute alcohol. The material has to be quite pure, however, and salt free. The procedure for crystallization from alcohol is as follows: Ribonuclease is first recrystallized twice by means of ammonium sulfate as described in the preceding section. 10 gm. of the crystal cake from the final crystallization is dissolved in 15 ml. of water and is dialyzed in a collodion bag for 24 hours against cold distilled water by the method of Kunitz and Simms (7). The dialyzed solution is made up with water to 50 ml., is cooled to about 5°C., and then 60 ml. 95 per cent alcohol of the same temperature is added with stirring. A heavy amorphous precipitate is formed which on standing in the cold room changes within several hours into a mass of fine fan shaped rosettes (Fig. 2) of rectangular or needle shaped crystals. The crystals are filtered with suction after 2 days, and washed several times with cold 95 per cent alcohol. They are dried for 24–48 hours in a desiccator over calcium chloride and then in the room for about 24 hours. The dry powder can be stored in a cool place indefinitely; yield is about 3 gm. of dry crystals.

II. Digestion of Yeast Nucleic Acid by Crystalline Ribonuclease

Addition of crystalline ribonuclease to a solution of yeast nucleic acid under appropriate pH and temperature conditions brings about a gradual splitting of the nucleic acid molecules into smaller components. This is shown by an increase in the diffusibility of the nucleic acid. The splitting

of the molecules of yeast nucleic acid by the new enzyme is accompanied by formation of titratable acid groups without the liberation of free phosphoric acid.

1. Diffusion through Collodion or Cellophane Membranes.—The striking difference in the diffusibility through cellophane between digested and undigested yeast nucleic acid is shown in Table I. The split products of digestion diffuse readily through membranes which are practically impermeable to the undigested acid. Similar results were obtained with collodion membranes.

2. Diffusion Measurements. An approximate estimate of the relative molecular size of the split products as compared with the size of the undigested yeast nucleic acid is conveniently obtained by measuring the diffusion coefficient of the material. The method of Northrop and Anson (8) has been employed for this purpose.

FIG. 2. Crystals of ribonuclease in alcohol. × 190

Experimental Procedure. 50 ml. 6.5 per cent yeast nucleic acid in 0.1 M sodium acetate pH 6.0 and containing 10 mg. of crystalline ribonuclease was left for several days at 5°C. until the maximum amount of digestion was reached. This solution and also a similar solution of nucleic acid but free of ribonuclease were then used for the diffusion experiment. The materials were allowed to diffuse into 20 ml. 0.1 M sodium acetate of pH 6.0 which was changed daily and analyzed for total phosphorus content.

The results are shown graphically in Fig. 3. The data for the diffusion coefficient at 5°C. of the digested as well as of the undigested yeast nucleic acid were plotted against the percentage of the material in the diffusion cell permitted to diffuse into the aqueous solvent. The graphs show that the

diffusion coefficient of the digested nucleic acid is practically constant until 50 per cent of the material has diffused and is numerically twice as great as the diffusion coefficient of the undigested acid.[4] If the assumption is made that the molecules of undigested acid, as well as of the split products, are spherically shaped, then the corresponding molecular volumes are approximately in the ratio of 8:1. The gradual decrease in the diffusion coefficient of the digested nucleic acid shows that the molecules of the split products are not all of the same size. The decrease may be due also to remnants of undigested nucleic acid.

TABLE I

Diffusion through Cellophane

Inside the cellophane bag: About 20 ml. 1.0 per cent yeast nucleic acid in 0.2 M borate buffer pH 7.0 and containing 7 mg. of ribonuclease. Control without enzyme.

Outside the bag: 30 ml. of same buffer solution without nucleic acid.

Left at 20°C. Samples analyzed for total phosphorus concentration.

	Control without enzyme		Digestion mixture	
	a	b	a	b
	Total phosphorus inside originally about 0.7 mg./ml.			
	mg. phosphorus per ml.			
Outside solution				
After 2.5 hrs.	Trace	0.0083	0.166	0.167
After 24 hrs.	0.059	0.089	0.37	0.24
Inside solution				
After 24 hrs.	0.59	0.69	0.32	0.33

3. Effect of Digestion on Precipitation with Glacial Acetic Acid. Undigested yeast nucleic acid is insoluble in concentrated acetic acid or in dilute hydrochloric or sulfuric acid; hence addition of these acids to a solution of yeast nucleic acid brings about complete precipitation of the nucleic acid. The effect of digestion of yeast nucleic acid by crystalline ribonuclease is to prevent the precipitation by acetic or other acids. This effect is very striking in the case of dilute solutions of yeast nucleic acid. Concentrated solutions of digested nucleic acid continue, however, to give precipitates when mixed with the precipitating reagents even after long digestion with an excess of enzyme. A quantitative study shows that the undigested material which is still precipitable amounts to 10–15 per cent of the total nucleic acid in solution. This may be due to the presence of some modified nucleic acid which cannot be attacked by the enzyme.

[4] Similar results were obtained by Dr. H. S. Loring. Personal communication.

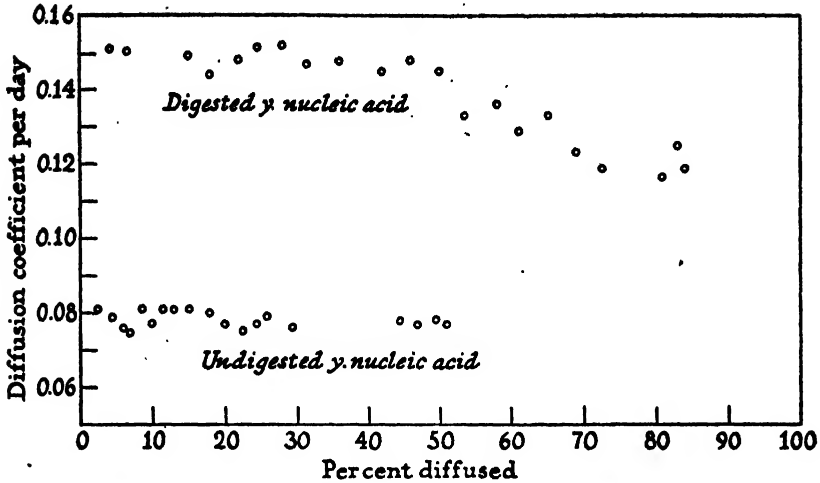


FIG. 3. Diffusion coefficient of digested and undigested yeast nucleic acid in 0.1 M sodium acetate pH 6.0 and 5°C.

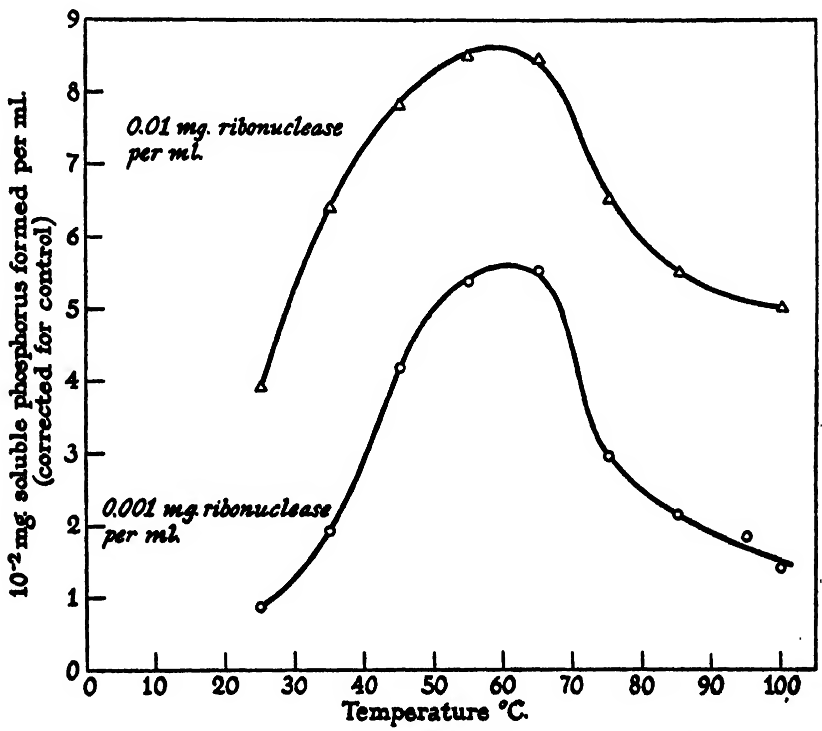


FIG. 4. Effect of temperature on the rate of digestion of yeast nucleic acid by ribonuclease.

(*a*) *Effect of Concentration of Enzyme.*—Tubes containing 0.5 ml. 2 per cent yeast nucleic acid in 0.1 M borate buffer pH 8.0 and 0.5 ml. of various concentrations of ribonuclease in water were placed in a water bath of 25°C. for 10 minutes. 10 ml. of glacial acetic acid was then added to each tube, mixed thoroughly, and filtered after 5 minutes through No. 42 Whatman paper. The total phosphorus per milliliter of filtrate was measured and designated as "soluble phosphorus." The results are given in Table II which shows that addition even of one part of ribonuclease to 2,000 parts of substrate causes formation of soluble phosphorus in 10 minutes at 25°C., equal to 73 per cent of the total phosphorus in the substrate. The ultimate extent of digestion even in the presence of a large excess of enzyme is 87 per cent.

(*b*) *Effect of Temperature.*—The optimum temperature for the rate of digestion as measured by the rate of formation of soluble phosphorus is

TABLE II

Digestion of 1 Per Cent Yeast Nucleic Acid by Crystalline Ribonuclease at 25°C.

Total phosphorus equals 0.75 mg. per ml. digestion mixture.

Mg. ribonuclease per ml. digestion mixture.......	0	0.005	0.05	0.5	5.0
Mg. "soluble phosphorus" formed in 10 min. per ml.........................	0.118	0.545	0.605	0.630	0.650
Per cent............................	16	73	81	84	87

65°C. as shown in Fig. 4. The rapid decline in the rate of digestion at temperatures above 65°C. is probably due to the inactivation of the enzyme.

Experimental Procedure.—Tubes containing 1 ml. yeast nucleic acid (0.5 mg. total phosphorus per milliliter) in 0.1 M acetate buffer pH 5.0 were placed for 3 minutes in water baths of various temperatures, then mixed with 1 ml. ribonuclease solution in water of 25°C. The digestion mixtures were left for 10 minutes at the various temperatures. The digestion was stopped by addition of 2 ml. uranium acetate reagent (see Methods). Control tubes containing mixtures of 1 ml. yeast nucleic acid and 1 ml. water were treated in the same manner as the digestion mixtures.

(*c*) *Effect of pH.*—Fig. 5 shows that ribonuclease acts best in the range of pH 7.0–8.2, the optimum being at pH 7.7.

Experimental Procedure.—1 ml. yeast nucleic acid (0.5 mg. total phosphorus per milliliter) in 0.1 M borate buffer of various pH plus 1 ml. ribonuclease, 0.0013 mg. per ml., in water. Final pH measured by means of a glass electrode. Digested 10 minutes at 25°C., then 2 ml. uranium acetate reagent added.

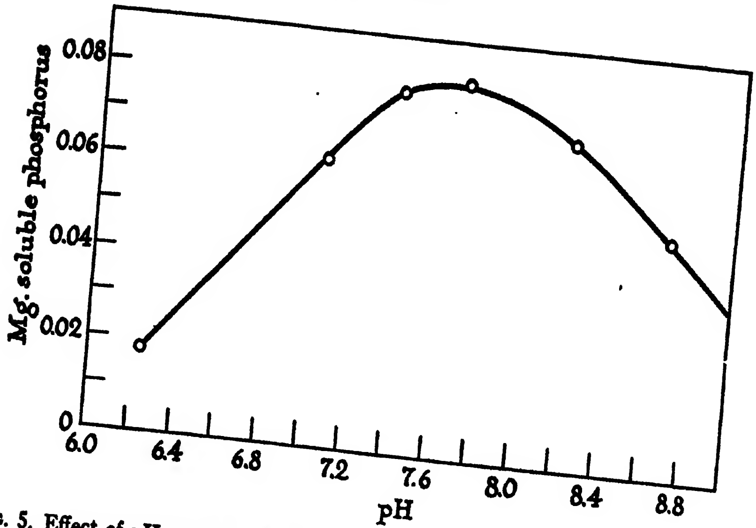


FIG. 5. Effect of pH on the rate of digestion of yeast nucleic acid by ribonuclease

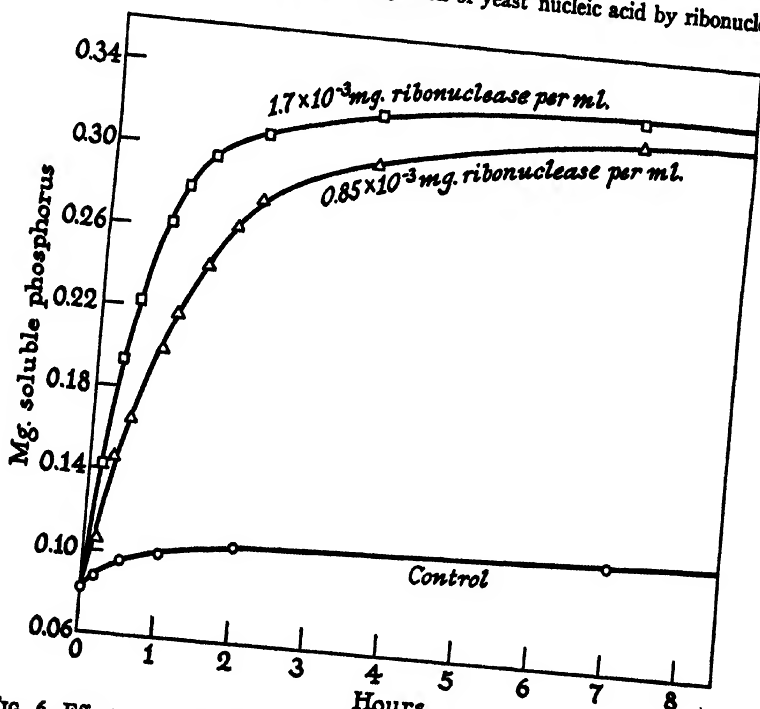


FIG. 6. Effect of concentration of ribonuclease on the rate of digestion of yeast nucleic acid.

(*d*) *Kinetics of the Reaction.*—The digestion of yeast nucleic acid by crystalline ribonuclease when measured by the rate of formation of soluble phosphorus follows the course of a typical enzymatic reaction.

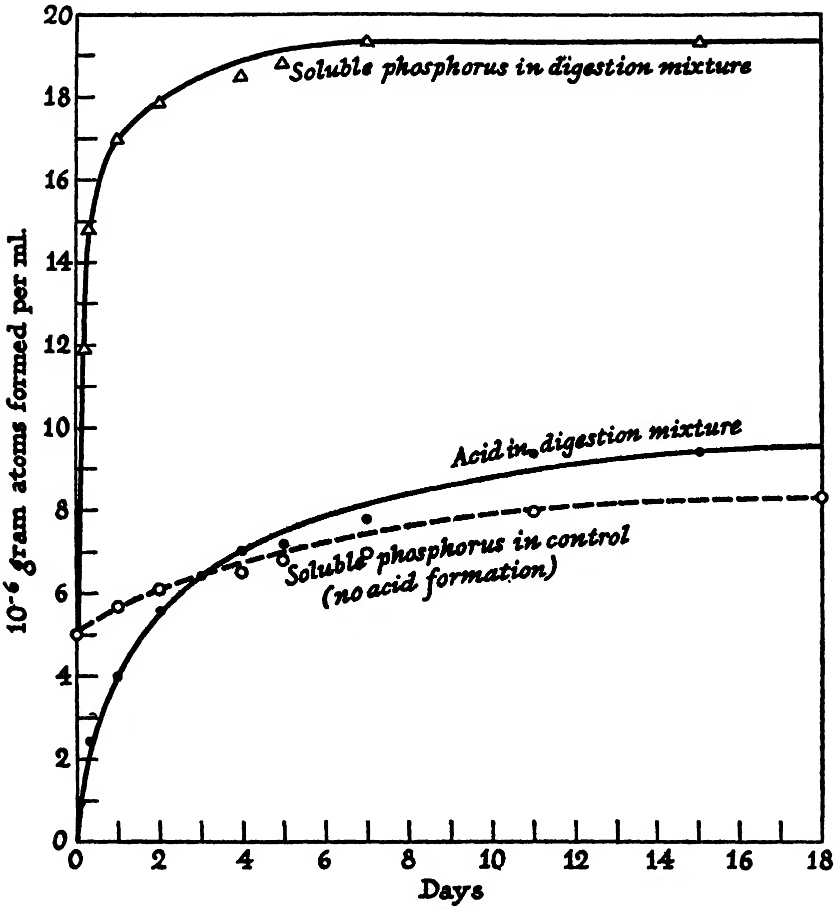


FIG. 7. Rate of formation of free acid

Experimental Procedure.—10 ml. yeast nucleic acid (0.5 mg. total phosphorus per milliliter) in 0.1 M acetate buffer pH 5.0 plus 5 ml. ribonuclease in water. Left at 25°C. Samples of 1 ml. plus 1 ml. 1 M hydrochloric acid, centrifuged after 10 minutes. Total phosphorus in supernatant measured.

Fig. 6 shows the effect of two different concentrations of ribonuclease on the rate of digestion. The time required for any amount of digestion is inversely proportional to the concentration of enzyme in solution while the ultimate amount of digestion is independent of the amount of enzyme used.

A mathematical analysis of the kinetics of the process is complicated by the fact that the enzymatic action is always accompanied by a significant amount of spontaneous hydrolysis of the substrate.

4. *Formation of Free Acid.*—The digestion of yeast nucleic acid by crystalline ribonuclease is accompanied by a gradual formation of titratable acid groups. The rate of formation of free acid is much slower than the rate of formation of soluble phosphorus as shown in Fig. 7. The ultimate amount of gram atoms of free acid formed is about one-half of the ultimate amount of gram atoms of phosphorus non-precipitable in concentrated acetic acid.

Experimental Procedure.—100 ml. 1 per cent yeast nucleic acid in 0.1 M acetate buffer pH 6.0, plus 2 ml. 0.1 per cent ribonuclease (or 2 ml. water in control). Left at 5°C. Samples of 1 ml. were mixed with 10 ml. glacial acetic acid for measurement of soluble phosphorus. At the same time samples of 5 ml. were pipetted into 50 ml. centrifuge tubes and titrated with 0.02 M sodium hydroxide to a definite pink color using 0.5 ml. 0.1 per cent phenolphthalein as an indicator.

III. Properties of Crystalline Ribonuclease

1. *Chemical and Physical Properties.*—Table III contains data for the elementary analysis and for other chemical and physical properties of ribonuclease. The material is a protein with a molecular weight of about 15,000.

2. *Stability.*—An aqueous solution of crystalline ribonuclease is quite stable over a wide range of pH when kept at temperatures below 25°C. Heating to higher temperatures causes gradual loss in enzymatic activity. The rate of inactivation varies, however, with the pH of the solution. The effect of heating solutions of ribonuclease at 100°C. is shown in Table IV. It is evident that ribonuclease is more stable in acid than in neutral or alkaline solutions. The region of maximum stability is between pH 2.0 and 4.5, as shown in Fig. 8.

Experimental Procedure.—Tubes containing 1 ml. 0.01 per cent ribonuclease in water, adjusted with acid or alkali to various pH (measured by a glass electrode) were kept in boiling water. The tubes were removed from the boiling water at various times, plunged for 1 minute in ice water, and left at room temperature for 30 minutes. The cooled solutions were then adjusted by means of acid or alkali to pH 4.0 for activity measurements.

3. *Change in Enzymatic Activity with Decrease in Native Protein.*—

(*a*) *Pepsin Digestion of Ribonuclease.*—Crystalline ribonuclease is readily digestible by pepsin in acid solution.

Experimental Procedure.—Pepsin digestion mixture—0.1 gm. crystalline ribonuclease, plus 9.5 ml. water, plus 0.15 ml. 5 M hydrochloric acid to pH 2.0, plus 1.5 mg. crystalline pepsin; left at 5°C. Samples of 0.5 ml. were brought to pH 9.0 by the addition of 4.5

TABLE III

Chemical and Physical Properties of Crystalline Ribonuclease

Elementary analysis in per cent dry weight (*a*)	C.	48.2
	H.	6.2
	N.	16.1
	S.	3.6 (partly inorganic)
	P.	Trace
	Residue.	0.1
Amino nitrogen as per cent of total nitrogen (*b*)		6.95
Tyrosine equivalent in millimols per mg. total nitrogen (*c*)		5.3×10^{-3}
Tryptophane (*d*)		0
Optical rotation of 5 per cent solution in water $[\alpha]_{25}^{D}$ per mg. nitrogen		−0.47
Molecular weight by osmotic pressure measurement (*e*) at 5°C. of 2.5 per cent solution in 0.5 M and 1 M ammonium sulfate (average of 9 determinations)		15,000 ± 1,000
Diffusion coefficient at 20°C. in 0.5 M ammonium sulfate by the method of Northrop and Anson (8)		0.092 cm.^{2} per day
Molecular volume calculated from diffusion coefficient		14,850
The following measurements were reported by Dr. Rothen (12)		
Isoelectric point by electrophoresis		About pH 8.0
Specific volume at 25°C.		0.707
Sedimentation constant at 25°C. in 0.5 M ammonium sulfate		1.84×10^{-13}
Molecular weight calculated from sedimentation and diffusion data		13,000
Diffusion coefficient in 0.5 M ammonium sulfate at 25°C.		0.116 cm.^{2} per day
Protein tests	Biuret	Positive
	Xanthoproteic	Positive
	Millon	Positive

(*a*) Analyses carried out at the Arlington Laboratories, Arlington, Virginia.

(*b*) Amino nitrogen measured by Van Slyke's manometric method.

(*c*) 1.0 ml. of dialyzed solution containing 0.13 mg. total nitrogen plus 1.0 ml. 1 M hydrochloric acid plus 3.0 ml. water plus 10 ml. 0.5 M sodium hydroxide plus 3.0 ml. of Folin and Ciocalteau's phenol reagent (9) diluted twice with water. Color read after 10 minutes against a similar mixture containing 1×10^{-3} millimols tyrosine.

(*d*) Colorimetric method of R. W. Bates (10).

(*e*) Method of Northrop and Kunitz (11).

ml. 0.2 M borate buffer pH 9.0. This stopped the digestion and also destroyed the peptic activity. The solutions were then analyzed for ribonuclease activity and protein content.

The results, as given in Fig. 9, show that the rate of digestion of ribonuclease protein by pepsin is accompanied by a corresponding percentage loss in the enzymatic activity of the ribonuclease.

(*b*) *Denaturation by Heat.*—The gradual inactivation of ribonuclease when heated at 100°C. is accompanied by gradual denaturation of the protein. The rate of denaturation can be measured by the change in the solubility of the protein in ammonium sulfate solution.

TABLE IV

Inactivation of Ribonuclease at 100°C.

pH	2.0 (0.01 M HCl)	3.5	5.8 (0.02 M acetate)	6.6	9.0
Time at 100°C.					
min.			*per cent activity left*		
5	93	95	64	7	0.3
15	87	87	44	2	0
30	78	79	29	1	0

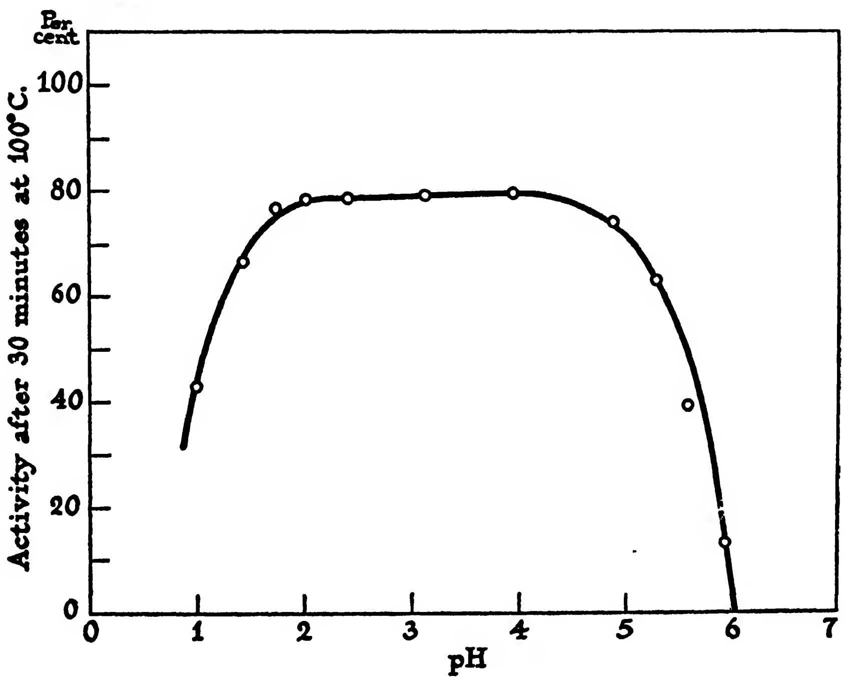


FIG. 8. Effect of pH on stability of ribonuclease at 100°C.

Experimental Procedure.—A series of tubes each containing 4 ml. 0.2 per cent ribonuclease in 0.02 M acetate buffer pH 5.8 was placed in boiling water. The tubes were removed at various intervals of time, cooled rapidly under running cold water, and allowed to stand at 20°C. for 5 minutes. Samples of 1 ml. of the cooled solutions were diluted with 0.01 M acetate buffer pH 4.0 for activity measurement, while samples of 2 ml. of the cooled solutions were mixed with 4 ml. of saturated ammonium sulfate for the

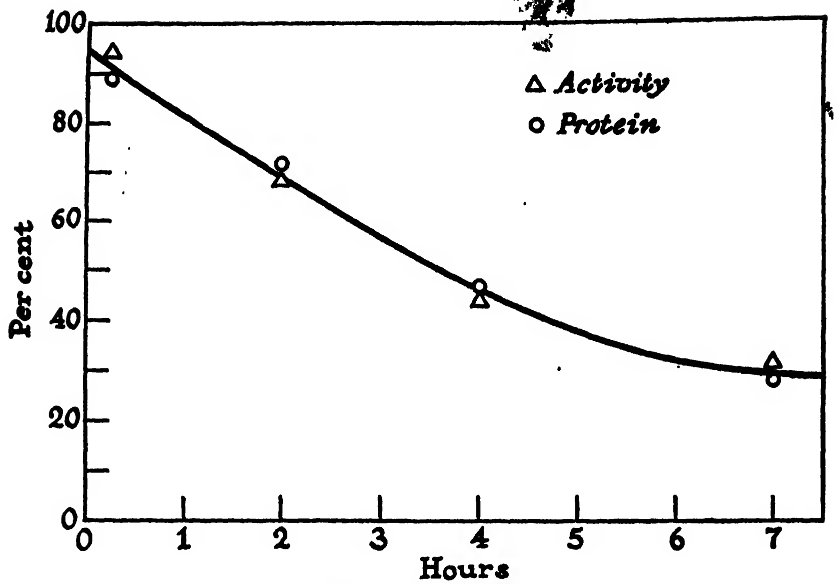


FIG. 9. Digestion of ribonuclease by pepsin

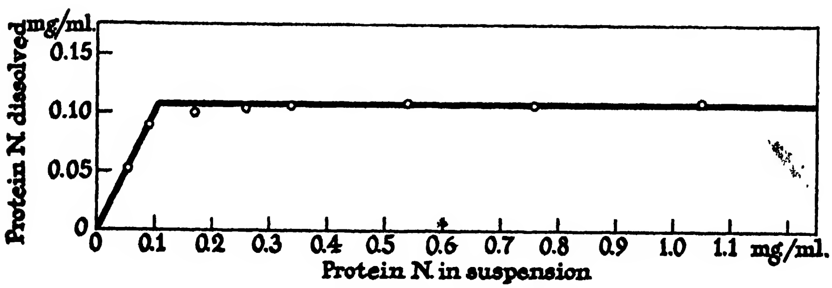


FIG. 10. Solubility of three times crystallized ribonuclease in 0.6 saturated ammonium sulfate pH 4.0 in the presence of increasing quantities of solid phase.

TABLE V

Denaturation of Crystalline Ribonuclease at 100°C.

Time at 100°C.	Activity	Native protein
min.	*per cent*	*per cent*
0	100	100
5	86	83
10	78	77
20	63	61
30	52	48

determination of the amount of denatured protein formed. The unheated sample of ribonuclease did not show any trace of precipitate in the ammonium sulfate mixture while the mixtures of the heated samples with 2 volumes of saturated ammonium sulfate solution gave rise to precipitates of denatured protein. The amount of precipitate formed increased with the time of heating of the sample at 100°C.

The ammonium sulfate mixtures were allowed to stand 5 minutes at 25°C. and then filtered through small No. 42 Whatman filter paper. The concentration of native protein in the filtrates was determined by precipitation in 10 per cent trichloracetic acid.

The results are given in Table V. The experiment shows that the inactivation of ribonuclease at 100°C. is accompanied by a corresponding proportional loss in the concentration of native protein in the ribonuclease solution.

(*c*) *Inactivation by Alkali.*—When ribonuclease is exposed to the action of alkali of pH 12 or higher it gradually loses its enzymatic activity. The loss in activity is also accompanied by a change of the native protein into denatured protein which, like the denatured protein produced by heat, is insoluble in 0.66 saturated ammonium sulfate. Experiments showed repeatedly that the rate of inactivation by alkali is proportional to the rate of change of the native protein into denatured protein.

It is thus evident that changes brought about in the protein molecule by various agents such as heat, alkali, or pepsin, are reflected in every case by a corresponding change in the enzymatic activity of the molecule. This suggests that the enzymatic activity is directly related to the protein molecule.

IV. Tests of Purity of Crystalline Ribonuclease

1. Repeated Crystallization.—Crystalline ribonuclease becomes relatively pure after two or three crystallizations and it retains through further repeated crystallization a constant activity per unit dry weight.

2. Fractional Crystallization.—The material after purification by two or three recrystallizations does not show any difference in the properties of the various crops of crystals obtained through fractional crystallization in various concentrations of ammonium sulfate. The specific activity of the first small crop of crystals does not differ from the specific activity of the succeeding crops and even from the specific activity of the last small amount of material left in solution in the mother liquor.

3. Solubility Test.—The theory as well as the technique of the solubility test for the purity of a protein has been described elsewhere (13). Measurements were made here of the solubility of crystalline ribonuclease in 0.6 saturated ammonium sulfate pH 4.0 in the presence of increasing amounts of crystals of ribonuclease in suspension.

Experimental Procedure.—The material used had been recrystallized three times and then washed several times at 20°C. with 0.6 saturated ammonium sulfate made up in 0.04 M acetate buffer pH 4.0 until the solubility of the crystals in the solvent became constant. Increasing amounts of a concentrated suspension of the crystals in 0.6 saturated ammonium sulfate pH 4.0 were made up in Lusteroid tubes[5] of about 20 ml. capacity, each provided with a Pyrex glass bead, to about 20 ml. with the same solvent. The tubes were stoppered with one-hole rubber stoppers and then plugged with short glass rods so as to remove all the air from the tubes. The suspensions were rocked for 24 hours and then centrifuged in an angular centrifuge[6] for 20 minutes at 3500 R.P.M. All operations, including the centrifuging, were done in a constant temperature room of 20°C. ± 0.5°. Samples of the total suspensions as well as of the clear supernatant solutions were analyzed for activity and protein nitrogen.

The results are shown in Fig. 10. The solid lines represent the theoretical solubility curve of a pure substance. The experimental points fall on the theoretical lines except for one or two points. This indicates the possible presence of a small amount of impurities in the material used. The analytical data for the concentration of protein nitrogen were used. The same result would be obtained if the activity data were used for plotting the curve since the ratio of activity to protein nitrogen was found to be practically constant in all cases.

4. Electrophoresis Test.—Ribonuclease after several recrystallizations does not show the presence of impurities differing in mobility from the bulk of material when tested by electrophoresis (12).

Methods

1. Estimation of Ribonuclease Activity.—Ribonuclease activity is expressed in terms of the rate with which the enzyme changes purified yeast nucleic acid into a form no longer precipitable either by acetic acid, by hydrochloric acid, or by a solution of uranium salt in trichloracetic acid. The last reagent, first suggested by MacFadyen (14), was found to give more reproducible results than acetic or hydrochloric acids. In general the measurements were confined to the initial stage of digestion where the effect is nearly proportional to the concentration of enzyme used. The rate of digestion is determined quantitatively by adding the precipitating agent to samples of the digestion mixture, filtering off the precipitate formed, and finally analyzing the filtrate for total phosphorus.

(a) Precipitation by Uranium Acetate in Trichloracetic Acid.—Yeast nucleic acid is precipitable from solution when mixed with an equal volume of 0.25 per cent uranium acetate in 2.5 per cent trichloracetic acid. The amount of precipitate is, however, decreased as the nucleic acid is digested by ribonuclease until it reaches a minimum of about 60 per cent in completely digested nucleic acid.

Experimental Procedure.—1 ml. of a solution of yeast nucleic acid (purified as de-

[5] Lusteroid Container Company, South Orange, New Jersey.

[6] Aktiebolaget Winkel Centrifuge, type S. P., Stockholm, Sweden.

scribed later) in 0.1 M acetate buffer pH 5.0 and containing 0.5 mg. total phosphorus is mixed with 1 ml. of ribonuclease solution containing from 0.001 to 0.01 mg. protein nitrogen in 0.01 M acetate buffer pH 4.0. The mixture is left for 10 minutes at 25°C. 2 ml. of uranium acetate is then added from a pipette, thoroughly mixed, left for 30 minutes at 25°C., and filtered through 7 cm. No. 42 Whatman filter paper. 2 ml. of filtrate, which is equivalent to 1 ml. of the original digestion mixture, is analyzed for total phosphorus content. This is designated as soluble phosphorus. The ribonuclease activity unit [N.U.] is defined as the activity which gives rise under these standard conditions to the formation of 1×10^{-3} mg. soluble phosphorus per milliliter of digestion mixture in a range of concentrations of enzyme where the amount of soluble phosphorus formed is proportional to the concentration of enzyme used. For convenience a standard curve is plotted, soluble phosphorus *vs.* [N.U.] from data obtained by measuring the activity of a series of dilutions of ribonuclease of a known enzyme content. The activity of any unknown solution of ribonuclease can then be determined from a single measurement by means of the standard curve. It was found generally that pure ribonuclease contains about 1000 [N.U.] per mg. protein nitrogen. This value varies considerably, however, with the sample of yeast nucleic acid used. The method for the measurement of activity was found also to be very sensitive to slight changes in pH of the substrate, as well as to the age of the solution. Hence, fresh solutions of nucleic acid have to be used and the pH carefully adjusted in order to obtain more or less reproducible results.

(*b*) *Precipitation by Glacial Acetic or Hydrochloric Acid.*—The procedure is the same as described in (*a*) except that 20 ml. of glacial acetic acid or 2 ml. 1.0 M hydrochloric acid instead of 2 ml. of uranium acetate reagent is added for the precipitation and the suspensions filtered after standing 5–10 minutes instead of 30 minutes.

The precipitation by acetic acid or by hydrochloric acid was found to be affected greatly by the presence of traces of ammonium sulfate and, in general, is less reproducible than the precipitation by the uranium acetate reagent.

2. Purified Yeast Nucleic Acid.—Commercial preparations of yeast nucleic acid were purified by reprecipitation with glacial acetic acid.

Experimental Procedure.—A suspension of 100 gm. commercial yeast nucleic acid in 500 ml. water was cooled in an ice water bath to about 2°C. 5 N sodium hydroxide was then added slowly until a clear solution was obtained. Care was taken to keep the solution cold and the pH not in excess of 6.0, as tested colorimetrically on a test plate. The volume of the solution was measured, 5 volumes of glacial acetic acid added, and the whole allowed to stand at 20–25°C. for 10 minutes. The precipitate formed was then filtered with suction on a large funnel, washed twice with about 100 ml. of water, and three times with 95 per cent alcohol. The dry precipitate, about 80 gm., was resuspended in 400 ml. water and treated as in the first precipitation. The final precipitate was washed with water, alcohol, and finally with ether, and dried in the air to constant weight. Final yield about 60 gm.

3. Total Phosphorus.—The colorimetric method of Fiske and SubbaRow (15) as modified by King (16) has been used.

4. Protein Nitrogen.—The ribonuclease protein was precipitated in 10 per cent trichloracetic acid. The amount of protein nitrogen was determined either by the turbidity method (17) or by the Kjeldahl nitrogen method (18).

The writer was assisted by Margaret R. McDonald and Vivian Kaufman.

SUMMARY

1. A crystalline enzyme capable of digesting yeast nucleic acid has been isolated from fresh beef pancreas.

2. The enzyme called "ribonuclease" is a soluble protein of albumin type. Its molecular weight is about 15,000. Its isoelectric point is in the region of pH 8.0.

3. Ribonuclease splits yeast nucleic acid into fragments small enough to diffuse readily through collodion or cellophane membranes.

4. The split products of digestion, unlike the undigested yeast nucleic acid, are not precipitable with glacial acetic acid or dilute hydrochloric acid.

5. The digestion of yeast nucleic acid is accompanied by a gradual formation of free acid groups without any significant liberation of free phosphoric acid.

6. Ribonuclease is stable over a wide range of pH even when heated for a short time at 100°C. Its maximum stability is in the range of pH 2.0 to 4.5.

7. Denaturation of the protein of ribonuclease by heat or alkali, or digestion of the protein by pepsin, causes a corresponding percentage loss in the enzymatic activity of the material.

LITERATURE

1. Jones, W., *Am. J. Physiol.*, 1920, **52**, 203.
2. Dubos, R. J., *Science*, 1937, **85**, 549.
3. Dubos, R. J., and Thompson, R. H. S., *J. Biol. Chem.*, 1938, **124**, 501.
4. Schmidt, G., and Levene, P. A., *J. Biol. Chem.*, 1938, **126**, 423.
5. Kunitz, M., *Science*, 1939, **90**, 112.
6. Kunitz, M., and Northrop, J. H., *J. Gen. Physiol.*, 1936, **19**, 1002.
   Northrop, J. H., Crystalline enzymes. The chemistry of pepsin, trypsin, and bacteriophage. Columbia Biological Series, No. 12, New York, Columbia University Press, 1939, 135.
7. Kunitz, M., and Simms, H., *J. Gen. Physiol.*, 1927–28, **11**, 641.
8. Northrop, J. H., and Anson, M. L., *J. Gen. Physiol.*, 1929, **12**, 543. Anson, M. L., and Northrop, J. H., *J. Gen. Physiol.*, 1937, **20**, 575.
9. Folin, O., and Ciocalteau, V., *J. Biol. Chem.*, 1927, **73**, 629.
10. Bates, R. W., *J. Biol. Chem.*, 1937, **119**, vii.
11. Northrop, J. H., and Kunitz, M., *J. Gen. Physiol.*, 1926, **9**, 354.
12. Rothen, A., *J. Gen. Physiol.*, in press.
13. Kunitz, M., and Northrop, J. H., in Cold Spring Harbor symposia on quantitative biology, Cold Spring Harbor, Long Island Biological Association, 1938, **6**, 325.
14. MacFadyen, D. A., *J. Biol. Chem.*, 1934, **107**, 299.
15. Fiske, C. H., and SubbaRow, Y., *J. Biol. Chem.*, 1925, **66**, 375.
16. King, E. J., *Biochem. J.*, London, 1932, **26**, 292.
17. Kunitz, M., *J. Gen. Physiol.*, 1938, **21**, 618.
18. Northrop, J. H., *J. Gen. Physiol.*, 1932, **16**, 335.

THE EFFECT OF OXIDANTS AND REDUCTANTS UPON THE BIOELECTRIC POTENTIAL OF NITELLA*

By L. R. BLINKS and M. J. PICKETT

(*From the School of Biological Sciences, Stanford University*)

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The surface of the living cell has so many unexplored properties, and is capable of responding electrically to so many changes in the environment (both ionic and otherwise), that it seemed desirable to test its response to oxidizing and reducing agents. This is especially significant, since it has been shown by many workers[1–9] that bioelectric potentials are influenced by oxygen tension (as well as by metabolic agents of other sorts); as a result, some theories[7, 8] have emphasized the relationship, or even the identity, of oxidation-reduction potentials with bioelectric potentials.

Oxidants and reductants have therefore been applied directly to several algal cells well adapted to bioelectric measurement. The results with *Nitella* are presented here.

Material and Method

The Californian *Nitella clavata* has been employed. *Nitella* has certain advantages for these studies; it can be exposed to low salt concentrations, (even to distilled water), as compared to marine plants such as *Valonia* and *Halicystis*, so that the full effect of oxidants or reductants can be observed, without possible interference by high salt

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[1] Mansfeld, G., *Arch. ges. Physiol.*, 1910, **131,** 457.

[2] Furusawa, K., *J. Physiol.*, 1929, **67,** 325.

[3] Gerard, R. W., *Am. J. Physiol.*, 1930, **92,** 498.

[4] Francis, W. L., *J. Exp. Biol.*, 1934, **11,** 35. Francis, W. L., and Pumphrey, R. J., *J. Exp. Biol.*, 1933, **10,** 379.

[5] Cowan, S. L., *Proc. Roy. Soc. London, Series B*, 1934, **115,** 216.

[6] Taylor, A. P., *J. Cell. and Comp. Physiol.*, 1935–36, **7,** 1.

[7] Lund, E. J., *J. Exp. Zool.*, 1926, **44,** 383; 1928, **51,** 265, 291, 327. Lund, E. J., and Kenyon, W. A., *J. Exp. Zool.*, 1927, **48,** 333. Lund, E. J., and Moorman, J. B., *J. Exp. Zool.*, 1931, **60,** 249. Lund, E. J., and Rosene, H., *Plant Physiol.*, 1935, **10,** 27.

[8] Marsh, G., *Plant Physiol.*, 1935, **10,** 681; *Carnegie Inst. Washington, Papers from Tortugas Lab.*, 1936, **31,** 1; 1939, **32,** 65; 1940, **32,** 99.

[9] Blinks, L. R., Darsie, M. L., and Skow, R. K., *J. Gen. Physiol.*, 1938–39, **22,** 255.

concentration (masking by strictly ionic effects, salting out of dyes, etc.). Buffering, which will be seen below as important, is also a simpler problem than in sea water, where calcium and magnesium tend to precipitate out in some buffers. The state of the cell can at all times be tested without changing solutions, by applying a sufficient voltage to initiate an action current. And the cells have been found remarkably uninfluenced by low oxygen tensions (in contrast to *Halicystis*,[9] and even to *Valonia*), so that highly reducing conditions can be maintained without complications due to the lack of oxygen itself.

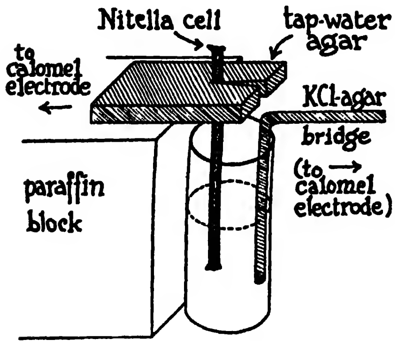

FIG. 1. Arrangement for holding *Nitella* cell, in cleft of agar block, the lower end dipping into the experimental solutions, which are changed by lowering and replacing the vial. KCl-agar bridges (the upper not shown) lead to calomel electrodes. A gold electrode was also sometimes inserted in the vial to measure the oxidation-reduction potential of the solution; and a fine tube led in hydrogen to reduce dye solutions (with platinized asbestos as catalyst), or air to re-oxidize them. The entire apparatus was covered with a bell jar, and a nearly saturated atmosphere maintained to prevent drying of cell.

On the other hand, with the external contacts employed, there is perhaps a little less certainty that the end of the cell immersed in the given oxidizing or reducing solution attains quite the conditions desired, since diffusion, and active protoplasmic streaming, may tend partly to equalize conditions at the two ends. An impalement technique, with the entire cell immersed in the given solution, is of some advantage here, and has been employed in the case of the other plants. In view, however, of the considerable length of cell immersed in the given solution, and the lack of any visible coloring along the cell when reduced dyes are applied to it, it is believed that this is not a serious difficulty.

The actual arrangement employed is shown in Fig. 1. An internodal cell of 5 cm. or more in length by 1 mm. diameter, was separated from adjacent cells some days before, and kept in pond water until used. One end was then mounted in a cleft of a soft agar block well soaked in pond water, and the lower end immersed for about 2 cm. in a vial of the desired solution. KCl-agar bridges made contact with distant calomel electrodes. Not shown in Fig. 1 are a fine tube for bubbling air or hydrogen, immersed in the vial, and in some cases a gold electrode for determining the oxidation-reduction potential of the solution. Usually the latter was determined immediately before or after application to the cell. The entire apparatus as shown, as well as the calomel electrodes, was covered between manipulations with a glass bell jar, within which a nearly saturated atmosphere was maintained to prevent drying of the cell.

Electrical measurement was by compensation with a potentiometer, using as null instrument a vacuum tube electrometer to draw no appreciable current. A stimulating potential (100 to 500 mv.) could be applied from the potentiometer by momentarily shunting the electrometer, and then opening it immediately to follow the course of the action potential and its transmission down the cell. Only cells which gave a charac-

teristic stimulation response were considered as normal. This usually persisted under all the treatments here reported, disappearing only after long exposures to the more toxic solutions (*e.g.*, safranin). Another test usually made after applying oxidants and reductants was exposure to NaCl or KCl of two concentrations, to see if the normal response to these still occurred. As shown in several of the figures, it was always obtained, even when, as usual, little or no response had been given to oxidants or reductants.

In addition, visual tests of condition such as appearance of plastids, protoplasmic streaming, and turgidity of the cell were made to assure healthy cells throughout. In general, the treatments, except with hydrosulfite as a reducing agent, were not injurious over the periods of exposure.

Usually the observed P.D. was close to zero, because with both ends of the cell intact, the equal and opposite potentials at each end cancelled each other. When there is a P.D., the sign is that of the solution at the end under treatment (lower end of cell, in vial), as measured in the electrometer circuit. That is, when the lower solution is positive, a positive current tends to flow outward across the protoplasm at that end, toward the measuring instrument. This is the usual convention in bioelectric measurements, but it should be noted that it is the opposite to that used in oxidation-reduction measurements, where the sign is that of the measuring *electrode*, not that of the solution in contact with it. Thus if the *Nitella* cell acted like a gold electrode, (which it does not in these experiments), it would tend to become positive in oxidizing solutions, making the outer solution, and hence the bioelectric potential, more negative; and *vice versa* with reducing solutions.

Room temperatures from 20 to 25° usually prevailed. Values in light and dark were not appreciably different, either with the cells or with electrodes, but bright light was avoided.

The E_h values given for the several solutions were observed at a gold, as well as at a platinum electrode, (against a saturated calomel electrode, but corrected to the normal hydrogen electrode by the customary factor, +0.245 v.). They are in fair agreement with the published E_0' values[10, 11] for the substances concerned, but any deviations are of no significance for the purpose in hand, as they are probably due to impurities which affect the fully oxidized or reduced substances as here used, much more than a 50 per cent mixture of oxidant and reductant.

Several different oxidation-reduction systems were employed, of which those reported here ranged from ferricyanide, with $E_h = +0.454$ v. to reduced safranin, with $E_h = -0.288$ v.,—a range of 0.740 v. Intermediate between these were: ascorbic and dehydroascorbic acid; ortho-chlorophenol-indophenol (oxidized and reduced); and indigo-disulfonate (oxidized and reduced). Most of these were employed around 0.001 M, although a few were more concentrated, as indicated in the figures. The exact molarity is questionable in the case of the dyes, because of unknown impurities (neutral salts, insoluble fractions, etc.), but is not important because the identical solution was always employed in both its oxidized and reduced state, being reduced by hydrogen plus pow-

[10] Michaelis, L., Oxidations-Reduktions-Potentiale, Julius Springer, Berlin, 1929.

[11] Hewitt, L. F., Oxidation-reduction potentials in bacteriology and biochemistry, London County Council, London, 4th edition, 1937.

dered platinized asbestos as catalyst, and reoxidized by air, often without removing the solution from contact with the cell.

A trace of the customary dye, 2–6 dichlorophenol-indophenol, was always added to the solutions of ascorbic acid or ascorbate, to indicate its state of reduction, and to serve as mediator at the electrode (possibly also at the cell surface) to aid electrometric measurement with this sluggish system.

All these solutions established definite and steady potentials at the gold electrode, returning to it after polarization, indicating adequate poising for the electrode, and presumably for the cell, although it is admitted that oxidation or reduction at the cell surface might occur, throwing off the value somewhat. However, the P.D. values with the cell were not appreciably different when the solutions were stirred (by bubbling with air or hydrogen) or remained quiet, indicating no great reaction at the surface.

One important precaution which had to be observed was adequate buffering of the solutions. It was found that without this buffering, rather prominent, but transient, changes of P.D. occurred on going from an oxidant to a reductant, and especially on aerating a reduced solution, although the P.D. later returned to its original level. (See Fig. 7.) These cusps disappeared, however, when the solutions were made up in dilute phosphate buffer, (usually 0.01 M), at pH 7.0. Evidently the changes of acidity accompanying oxidation or reduction of a dye, etc., were affecting the cell, rather than the change of E_h as such, which, of course, still changes on oxidation or reduction in the presence of buffers—indeed the more reproducibly and stably. Since buffering is a standard practice in measurements with electrodes, this but further emphasizes the difference between the latter and the cell surface.

A related precaution, and one which is also largely obviated by using buffers, concerns the number of corresponding K or Na ions, etc., associated with an oxidized or reduced anion. An example is shown below in the case of ferri- and ferrocyanides, where three and four K ions are respectively involved in a given molarity. These in themselves cause a change of P.D. in *Nitella* (though not at an electrode), which has to be corrected by using a 4 to 3 ratio of molarity in the two cases. The effect can also be corrected by adding 1/3 the concentration of KCl to the ferricyanide solution. Where dyes were employed, the buffers were sufficiently more concentrated so that this valence effect was less significant.

EXPERIMENTAL RESULTS

The effects produced by potassium ferro- and ferricyanide are shown in Fig. 2. It might be thought that these salts would be toxic, but the cyanide is already in an iron complex, and no injurious effect seems to occur. (Nor is any trace of the salt detected in the vacuole after several hours exposure, using the very delicate ferri- and ferro-ion color tests. This agrees with the apparently low ionic mobility of the ferro- and ferricyanides, as noted below.)

A prompt shift of some 80 mv. is first seen on changing from pond water to 0.005 M K_4 ferrocyanide, but this may be attributed to the increase of K ion concentration, since KCl of the equivalent K concentration (0.02 M), maintains the same P.D. when substituted later. A similar explanation

accounts for the change of P.D. when 0.005 M K_3 ferricyanide is substituted for the 0.005 M ferrocyanide; the P.D. becomes less negative, but is restored to essentially the same value when the concentration is increased to

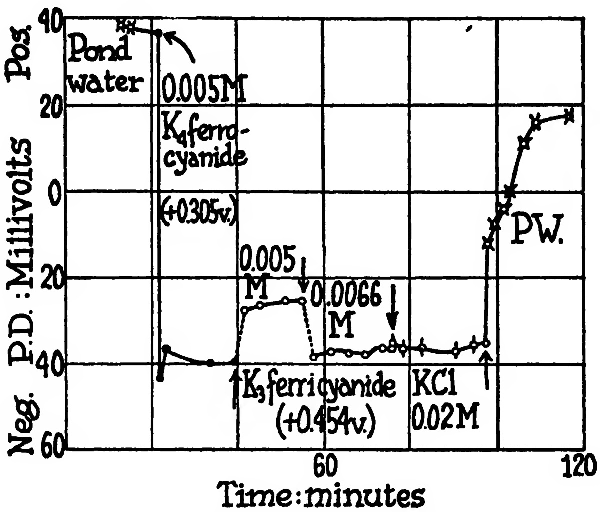

FIG. 2. Effect on P.D. of applying potassium ferro- and ferricyanide to *Nitella* cell as compared with KCl of equivalent K concentration. There is an initial positive P.D. of some 40 mv. between the two ends of the cell, when both are in pond water. On application of 0.005 M K_4 ferrocyanide to the lower end, the P.D. shifts by 80 mv., to 40 mv. negative. Changing to 0.005 M K_3 ferricyanide drives the P.D. less negative by some 15 mv., but this is due to the decreased K concentration; when the latter is corrected by using 0.0066 M K_3 ferricyanide, the P.D. is essentially the same as in ferrocyanide, although the E_h values of the two are 150 mv. apart. Substitution of 0.02 M KCl also maintains the same P.D. Restoration of pond water (P.W.) restores the P.D. toward its original value (not always immediately reached after K exposures).

The sign of the P.D. is that of the lower, experimentally changed solution; a positive P.D. tends to produce positive current outward across the protoplasm toward this solution, and thence toward the electrometer. Figures in parentheses (E_h values in volts) represent the oxidation-reduction potential of the solutions, as measured at a gold electrode in contact with them. Arrows show time of changing solutions.

All solutions (except the pond water) were made up in 0.01 M Na phosphate buffer, at pH 7.0.

0.0066 M, bringing the K ion concentration to 0.02 N. (The higher activity coefficient of ferricyanide[10] may also influence the effect.)

It should be pointed out that this small correction for the K ion concentration produces no significant shift in the E_h values of either ferro- or ferricyanide. Furthermore, the bioelectric difference between *equal* molarities of the two salts is in the *opposite* direction to their effect upon an

electrode; ferricyanide makes the electrode more positive, while it makes the cell more negative (or the outer solution more positive, in the bioelectric convention).

In general, when the K concentration is kept constant by making similar adjustments of the total concentrations of salts, then all K salts give much the same P.D. (Fig. 3), whether they are ferri- or ferrocyanide, chloride, sulfate, etc. Evidently the anion is a matter of little importance, being so slightly mobile in all cases that the cation determines the P.D. In line with this, Na ferri- and ferrocyanides give less P.D. change than the K salts for a given concentration, which agrees with the lower mobility of the Na ion, while the oxidation-reduction potential of Na salts is of course essentially the same as that of K salts.

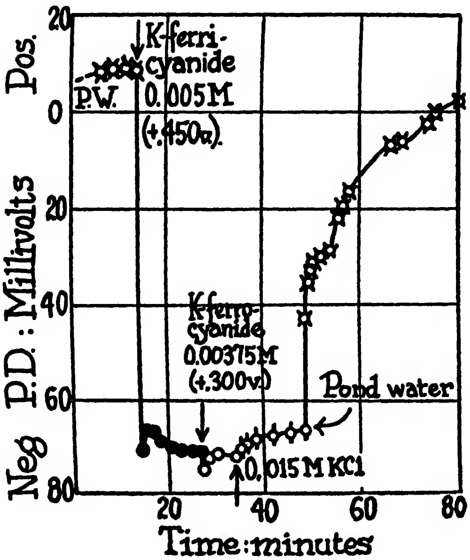

FIG. 3. Similar effects of K ferro- and ferricyanides,and of KCl, when the K ion concentration is adjusted to be the same in each case (here K = 0.015 M). The large change of P.D. on replacing pond water with 0.005 M K_3 ferricyanide, is scarcely altered on changing to 0.00375 M K_4 ferrocyanide, nor from that to 0.015 M KCl. (All these solutions made up in 0.01 M Na phosphate buffer at pH 7.0.) The original value is nearly regained on restoration of pond water (twicc renewed to wash away traces of KCl).

Designations as in Fig. 2.

A similar indifference to the state of oxidation, here of a natural plant product, ascorbic acid (vitamin C, cevitamic acid Merck) is seen in Fig. 4. Here 0.005 M ascorbic acid, neutralized just before with NaOH, and buffered with phosphate buffer at pH 7, establishes essentially the same P.D. on the cell as the buffer itself, or the ascorbate well oxidized by long air bubbling or exposure to H_2O_2. As is well known, only the ascorbic acid establishes a stable potential at the gold electrode, the more oxidized forms, *e.g.*, dehydroascorbic acid being poorly reversible.[12] The latter must therefore be regarded as furnishing indifferent ions, comparable to

[12] Borsook, H., Davenport, H. W., Jeffreys, C. E. P., and Warner, R. C., *J. Biol. Chem.*, 1937, **117**, 237. King, C. G., in Cold Spring Harbor symposia on quantitative biology, Cold Spring Harbor, Long Island Biological Association, 1939, **7**, 137.

chloride or sulfate, etc., valuable here chiefly to maintain an equivalent ion concentration, their more positive potential being due to impurities (iron, etc.). But the well poised, reducing ascorbate ion evidently has

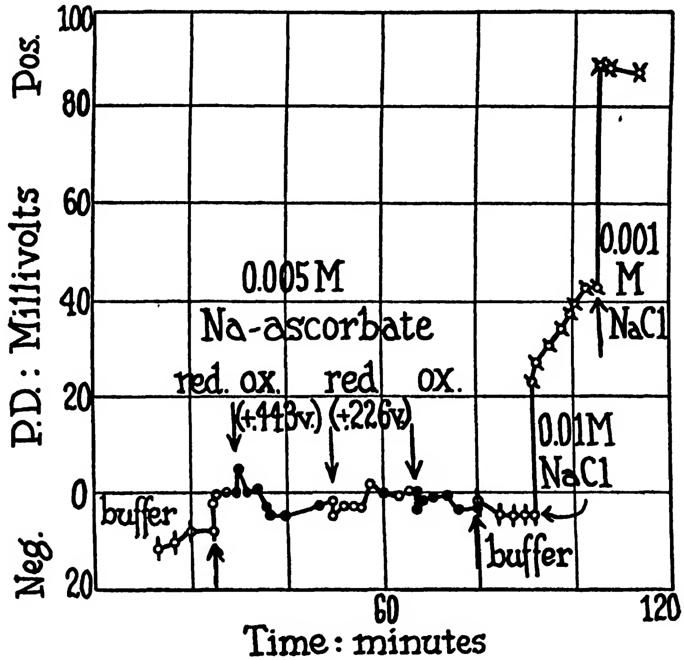

FIG. 4. Effect of normal (reduced) and oxidized Na-ascorbate of the same concentration (0.005 M), made up in 0.01 M Na phosphate buffer at pH 7. The P.D. is first shown with the lower end of the cell in contact with the buffer alone; then the reduced ascorbate is added, with a small shift of P.D. This is scarcely altered when the oxidized form is substituted, nor on repetition of these two exposures. On the contrary, when the buffer is replaced by 0.01 M NaCl, and this in turn by 0.001 M NaCl, large changes of P.D. are produced.

The reduced ascorbate was made by neutralizing ascorbic acid (vitamin C, cevitamic acid, Merck) with NaOH. Half the sample was then oxidized with H_2O_2, the latter boiled off, and reneutralized with NaOH. The oxidized sample probably was a mixture of the several oxidation products of ascorbic acid, but did not reduce 2–6 dichlor-phenol-indophenol, a trace of which was present to indicate the state of reduction. The E_h values given were observed at a gold electrode, the poising of the oxidized form probably being due to impurities and the dye.

no more electrical effect upon the cell than have these indifferent, unpoised oxidized products, although their apparent, or effective E_h lies over 200 mv. apart.

Turning now to dyes for more reducing systems, *Nitella* displays the same indifference to oxidized and reduced ortho-chloro-phenol-indophenol,

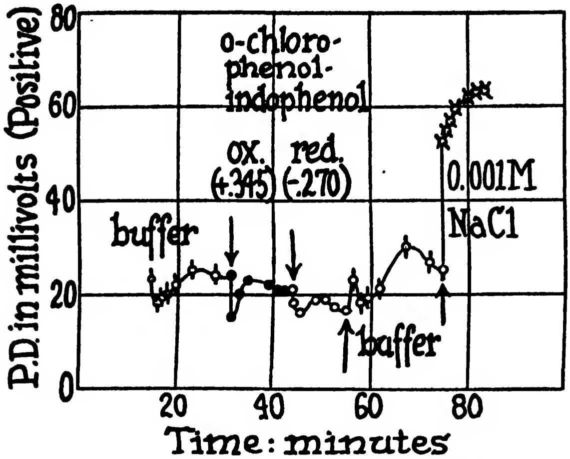

FIG. 5. Effect of ortho-chloro-phenol-indophenol, oxidized and reduced forms, used ½ saturated (approximately 0.001 M) in 0.005 M Na phosphate buffer at pH 7.0. There is no very significant difference when either of these forms is applied, compared to the buffer alone, in which the P.D. is also somewhat unstable. This is despite the difference of 615 mv. in the E_h of the oxidized and reduced forms (the latter reduced by bubbling hydrogen, with powdered, platinized asbestos as the catalyst, suspended in solution). On the other hand there is a good response to substitution of 0.001 M NaCl for the buffer.

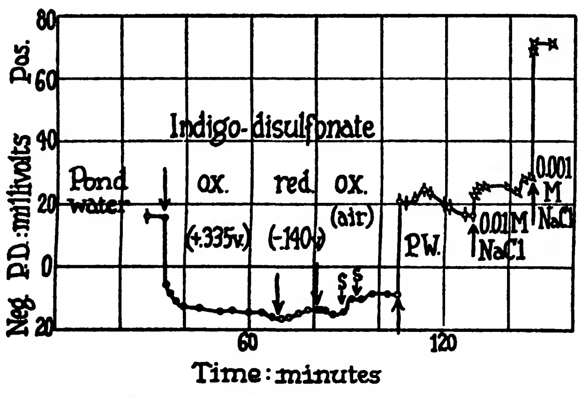

FIG. 6. Effects of 0.001 M indigo-disulfonate, in oxidized and reduced form, on the P.D. of *Nitella*. The dye, dissolved in 0.01 M Na phosphate buffer at pH 7.0, was applied at the first arrow, making the P.D. about 30 mv. more negative than in pond water, because of the more concentrated salts present. The leuco-form (reduced by hydrogen plus platinized asbestos) was then applied, with no significant change, despite the 475 mv. shift in oxidation-reduction potential. Nor was there a change on re-oxidation (by aeration). Two test stimulations, (marked *S*) gave good responses (not shown) and slightly decreased the P.D.

In contrast, large changes of P.D. occurred on restoring pond water (P.W.), and on changing from 0.01 M NaCl (about equivalent to pond water here) to 0.001 M NaCl.

dissolved in phosphate buffer, and used in the oxidized, colored form, as well as the reduced, leuco-form. Aside from initial cusps (which frequently occur with *Nitella* on any change of solution, probably due to varying length of the cell exposed to solution, etc.) the P.D. is within a few millivolts with each form, although the E_h as measured on the gold electrode was +0.345 v. for the oxidized, and −0.270 v. for the reduced form. (Traces of impurities may have poised this reduced dye at the low value found, the E_0' being accepted as +0.233 v. at pH 7.) Neither is it essentially different with the plain buffer, although a certain amount of drifting is evident in all these solutions in Fig. 5. Compare, however, the much larger P.D. change produced on going from 0.005 M phosphate buffer, to 0.001 M NaCl, at the end of Fig. 5, showing the usual large ionic concentration effect.

Similar indifference to an oxidized and reduced dye is shown in Fig. 6 for indigo-disulfonate (in phosphate buffer). Here the P.D. is almost identical when the E_h is changed from +0.335 v. to −0.140 v. by reducing the dye with hydrogen plus platinized asbestos, or on re-oxidizing it with air. On the other hand a good ionic concentration effect is again obtained on tenfold dilution of NaCl, and the cell stimulates normally at the 2 points marked *S*.

The most negative dye employed, safranin-O, is also the most toxic for *Nitella* cells, staining their walls heavily, and eventually rendering them soft and impossible to stimulate. However, over short periods, and even up to an hour or more, it produces little effect upon the bioelectric potential, whether in the oxidized or the leuco-form. Fig. 7 shows an example, not with the usual buffer, but in pond water and distilled water, showing especially with the latter the cusp that is frequently found on aerating an unbuffered leuco-dye. However, the earlier oxidations and reductions, whether of 0.0001 or 0.001 M safranin, produce very small effects compared to the ionic concentration effect on tenfold dilution of NaCl.

Finally, a *mixture* of two dyes was employed, one rather negative, one positive in E_0' to obtain somewhat better poising at extreme oxidation and reduction. This consisted of equal concentrations of safranin and *o*-chlorophenol-indophenol in phosphate buffer. The bioelectric potential changed by less than 5 mv. when this mixture was reduced, and the direction of the change was in the opposite direction to that expected at a gold electrode, the solution becoming more negative instead of more positive with respect to the cell (Fig. 8). The entire change may have been fortuitous, a small amount of drifting P.D. often being found with *Nitella*. On re-oxidation of the same solution (by aeration) there was essentially no change of P.D. despite a total E_h change of 643 mv. On the other hand substitution of three different concentrations of NaCl gave good concentration potential

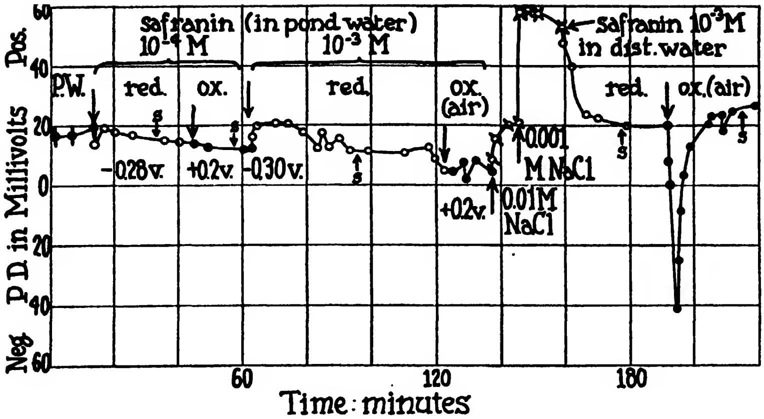

FIG. 7. Effects of safranin-O, reduced and oxidized, on P.D. of *Nitella*. In this case the dye was dissolved, not in the customary phosphate buffer, but in either pond water or distilled H_2O. On changing from pond water (P.W.) at the start, to reduced safranin (0.0001 M) in pond water there is a slight cusp but no further change, nor is there on oxidation by air. The concentration is then increased to 0.001 M, and except for irregularities, much the same P.D. is maintained, as it also is on aeration. On the contrary there is a much greater change on substitution of 0.01 M and 0.001 M NaCl.

0.001 M safranin, dissolved in distilled water is then applied, first reduced, then oxidized (by air). The large cusp occurring at the latter change is characteristic in distilled water, but is usually missing in buffered solutions. It is probably due to acidity changes; see text. Several stimulations were given at points marked *S*, with monophasic or diphasic action currents resulting (not shown) and indicating a normal condition of the cell.

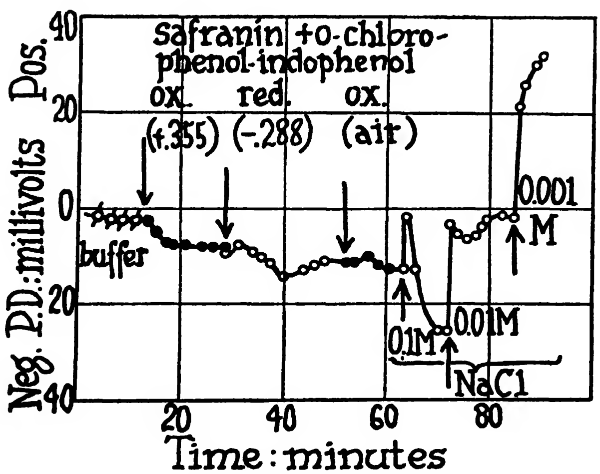

FIG. 8. Effect of mixture of safranin and *o*-chloro-phenol-indophenol on P.D. of *Nitella*. The dyes, each about 0.0005 M, were dissolved in 0.01 M Na phosphate buffer at pH 7, and were employed to give better poising at both oxidized and reduced ranges than the single dyes. There is again almost a complete independence of the P.D. on the degree of oxidation or reduction, despite a 663 mv. difference of E_h. On the contrary, good P.D. changes accompany the dilution of NaCl, at the end of the record.

changes. Indeed, here, as throughout the experiments, *Nitella* proves itself a much better K or Na "electrode" than it does a gold or platinum one.

DISCUSSION

It is to be concluded that the outer surface of *Nitella* does not act like an indifferent electrode, of platinum or of gold, etc., to give manifest potentials by reversible electron donation or acceptance with the several oxidation-reduction systems here employed.[13] Whether other substances, more "natural" or biological in character might be more effective, due to some specificity of enzymes or mediators in the cell surface, is not yet evident. But the question could be answered by this type of direct experiment and will be attempted with other substances, as available. Some of these, such as cytochrome, the yellow enzyme, etc., could probably not reach the outer surface of a plant cell because the cellulose wall would hold back the protein "bearer." But they can be perfused in the vacuole, and such experiments will be reported on *Halicystis*, where in general much the same indifference has been found to the substances here used. Complications occur in both *Halicystis* and *Valonia* caused by changes due to low oxygen tension as such,[9, 14] quite independently of a low oxidation-reduction potential in the medium, but technically impossible to prevent with the more reducing substances. This makes the answer more restricted; but it may be said that, under the conditions set by this characteristic, namely a cell surface definitely altered to strictly ionic effects, there is again an indifference to the degree of oxidation or reduction of a given substance, within a fairly wide E_h range.

There are, however, certain changes in these other cells produced by the more oxidizing agents, which might on first glance be taken for an electrode-like response, but which seem referable to an indirect effect of the oxidant, possibly *via* an increased acidity. This will be reported elsewhere. It would indeed be surprising if the cell surface, often assumed to be lipoid, and possibly unsaturated was *not* altered by strongly oxidizing or reducing substances. But most of these lie outside the range here reported, and that generally prevailing in cell media or interiors.

Unanswered by these experiments, and possibly unanswerable save by

[13] For a recent consideration of the conditions which might make oxidation-reduction potentials electrically manifest, either in artificial or biological membranes, see Korr, I., in Cold Spring Harbor symposia on quantitative biology, Cold Spring Harbor, Long Island Biological Association, 1939, **7**, 74 (including Discussion, p. 91). The important biological question is not the theoretical possibility of such membranes, or their artificial preparation, but their actual demonstration in living cells, by direct methods such as those attempted in the present paper.

[14] Blinks, L. R., *J. Gen. Physiol.*, 1939–40, **23**, 495.

greatly refined micrurgical technique,[15] is the question whether other membranes or phase boundaries *within* the cell can respond with a manifest potential to oxidants or reductants in contact with them. However, such surfaces would have to be practically continuous, and electrically unshunted (or slightly shunted) to give appreciable potentials. The chief membranes of this sort known in cells are the outer, and in plants the vacuolar, surfaces. Since the outer surface responds well to many ionic changes, and can be metabolically altered in its response to these, an adequate explanation for most bioelectric phenomena seems to be at hand in its known properties without postulating other membranes of unknown location.

SUMMARY

Nitella cells were exposed to various oxidants and reductants, to determine their effect upon the bioelectric potential. These included five systems, with an E_h range from +0.454 v. to −0.288 v., a total range of 0.742 v.

When proper regard was given to buffering against acidity changes, and concentration changes of Na or K ions in the oxidized and reduced forms, no significant effect upon the bioelectric potential was found:

1. When an oxidant or reductant (K ferri- or ferrocyanide) was applied instead of an equivalent normality of an "indifferent" salt (KCl).

2. In changing from a given oxidant to its corresponding reductant (ferri- to ferrocyanide; oxidized to leuco-dye, etc.).

3. When a mixture of 2 dyes, (indophenol with positive E_0', and safranin with negative E_0') was oxidized and reduced, to give better poising at the extremes.

It is concluded that the outer surface of this cell is not influenced by the state of oxidation or reduction of the systems employed; at least it does not respond with a manifest change of bioelectric potential to changes in oxidation-reduction intensity of the medium.

The cells continued to show, however, at all times their usual response to concentration changes of KCl, NaCl, etc., and to electrical stimulation.

[15] An experiment of Umrath (Umrath, K., *Protoplasma*, 1933, **17**, 258) was directed toward the opposite question, namely, whether there was a change of oxidation-reduction potential within the protoplasm when the bioelectric potential underwent a change. He employed two contacts within the protoplasm of *Nitella*, one a platinum electrode, the other a micro-salt-bridge leading to a distant reversible electrode. On stimulating the cell, little or no change of potential occurred between these two contacts, indicating no change of internal oxidation-reduction potential, although the usual large action potential was picked up by an external electrode. This is the complement of the experiments here reported: a large bioelectric change, accompanied by no change of E_h within the protoplasm.

STUDIES ON PHOTOSYNTHESIS

Some Effects of Light of High Intensity on Chlorella*

By JACK MYERS and G. O. BURR

(*From the Department of Botany, University of Minnesota, Minneapolis*)

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INTRODUCTION

It is the purpose of this report to present quantitative data on some effects of high light intensities on *Chlorella*. No comparable data have as yet been published, although a number of workers, using various techniques, have studied the effects of intense light on the green plant. It has been generally demonstrated that after long exposure of a plant to strong light there may be a disappearance of the products of photosynthesis (solarization), a decrease in the apparent rate of photosynthesis, or microscopically observable injury to the tissue. In this paper the term solarization will be used to indicate a reduction in photosynthetic rate due to prolonged exposure to light.

The literature has been reviewed in the more recent work of Emerson (1935), Fockler (1938), Holman (1930), and Stålfelt (1939) and need not be considered in detail here. Most of the work has dealt qualitatively either with the ecological aspects of the problem or with the mechanism by which the observed effects might be brought about. The data to be presented in this paper describe the solarization effect as a function of light intensity and time.

EXPERIMENTAL

As experimental material there were used cultures of *Chlorella vulgaris*,[1] of *Protococcus sp.*[1, 2] and of *Chlorella pyrenoidosa*.[3] These have been grown in a ⅙ Detmer solution

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[1] Identified by Professor Felix Mainx of the German University at Prague. Obtained through the courtesy of Dr. C. E. Skinner by whom they had been isolated from soil.

[2] *Protococcus* was used only to support data obtained with *Chlorella*. A comparatively low rate of photosynthesis makes this organism more difficult to work with.

[3] Obtained through the courtesy of Dr. Robert Emerson.

as recommended by Miss Meier (1934), with and without the addition of glucose. Cultures were grown in 500 cc. Erlenmeyer flasks in darkness and in light with either air or 5 per cent CO_2 in air bubbled through. To obtain darkness flasks were wrapped in photographic light-proof paper and kept in covered iron pails. Cultures grown in light were placed uniformly around the 5.5 cm. water jacket surrounding a 300 watt bulb. The intensity at the illuminated side of the flasks was approximately 450 foot-candles.

Sterile precautions were observed only when glucose was added to the nutrient solution, although microscopic tests for contamination were made in all cases.

Measurements were made by the familiar Warburg technique, using one illuminated experimental vessel with flat bottom and a non-illuminated thermobarometric control. The volume of the experimental flask was 14.22 cc. to the level of Brodie fluid as calibrated with mercury. When used as described below 1 mm. increase in pressure corresponds to an oxygen evolution of 0.56 c.mm.

The constant temperature bath was held at 26°C. ±0.01° as checked by a Beckmann thermometer. High light intensities were obtained by an optical system mounted horizontally beneath the bath.[4] The light of a projection bulb was condensed by two planoconvex lenses each of 6 inches diameter and 7 inches focal length. The horizontal beam was then reflected vertically upward through the glass bottom of the bath by a concave mirror. The area of the light beam in cross-section at the level of the experimental flask was large enough so that the flask was always illuminated during the 3 cm. amplitude of its shaking cycle. The light thus passed through 8 inches of water before reaching the flask and most of the infrared was removed. Various intensities were obtained by the use of three different projection bulbs and a series of screens. For higher intensities, a Westinghouse or General Electric 1000 watt projection bulb with a C13D filament was used. For lower intensities, projection bulbs of 250 or 500 watts were sufficient. Light intensities of over 30,000 foot-candles could be obtained with new 1000 watt projection bulbs. Unfortunately these bulbs have a rather rapid decay and a short average life.

Light intensities were measured by a Weston photronic cell, rigidly mounted immediately over the bath and in the light beam above the experimental flask. This was wired to a calibrated micro-ammeter. The light intensity falling on the bottom of the experimental flask was found to be 1.7 times as great as the intensity of the photronic cell in its fixed position at which all readings were taken. This factor was therefore used as a constant correction. Readings were taken at the beginning and end of each run, when the experimental flask was not in the light beam, and the average value considered representative. Changes in line voltage never caused more than 5 per cent variation in the two readings.

Most of the experiments to be considered were carried out in a potassium carbonate-bicarbonate buffer corresponding to the sodium carbonate-bicarbonate buffer No. 9 of Warburg (1919) (0.015 M K_2CO_3, 0.085 M $KHCO_3$). According to the calculations of Smith (1937) from the data of MacInnes and Belcher (1933) this gives a CO_2 concentration of 78.7×10^{-6} M. In one experiment buffer No. 11 was used (0.005 M K_2CO_3, 0.095 M $KHCO_3$ = 290×10^{-1} M CO_2). The buffer method was used because of its simplicity and higher degree of accuracy. Pressure changes are due entirely to oxygen

[4] Essentially similar to that described by Smith (1937).

uptake or evolution. No harmful effects of the buffer have ever been observed, even though suspensions have been kept in it in the icebox for as long as 24 hours.

In several check experiments the cells were suspended in ⅛ Detmer solution saturated with 5 per cent CO_2 and the gas space swept out with 5 per cent CO_2. The use of nutrient solution depends on the differences in solubilities of carbon dioxide and oxygen. Interpretation of such results requires a knowledge of the photosynthetic quotient, which would be difficult to obtain with the limited cell volumes used. Hence only a small number of these runs were made.

Immediately before an experiment the cells were centrifuged out of the nutrient solution, taken up in the buffer, and centrifuged out again in a graduated tube. To the packed cells fresh buffer was added to give a suspension in which each 1 cc. contained 0.01 cc. of cells. The suspension was then kept in the dark except when aliquot portions were withdrawn. When the cells were grown in darkness, all further operations except the reading of cell volume were performed in the dark. Aliquot portions were then withdrawn in the dark by an automatic pipette. When nutrient solution was used in place of the buffer, the stock suspension was made up in ⅛ Detmer solution.

1 cc. of the cell suspension (containing 0.01 of cells) was added to 7.2 cc. of buffer in the experimental flask. The flask and manometer were placed in position without illumination and about 5 minutes allowed for adjustment to equilibrium. The "zero" reading was then taken in the dark. The light was immediately turned on and succeeding readings taken every 5 minutes. A jump observed between the 0 and 5 minute readings (and in fact the pressure change accompanying any change in light intensity) is therefore largely an instrumental error due to readjustment of temperature equilibrium. The above procedure was adopted, in spite of the inherent error in the first two readings, because of its reproducibility.

The temperature increase within the flask caused by light absorption as referred to above is negligible in its effect on the physiological processes involved. The temperature increase produced by 28,000 f.-c. in a shaken flask containing eight times the usual number of algal cells was only about 0.4°C. as measured by a mercury thermometer. The Warburg instrument must always be considered as a very sensitive gas thermometer.

The authors consider of utmost importance the fact that only very dilute cell suspensions were used. This reduces shading of cells to a minimum not obtainable with any other combination of materials and methods, with the possible exception of that of van den Honert (1930) and van der Paauw (1932) who used a single layer of *Hormidium* cells. More dilute suspensions cannot be made without great reduction in the accuracy of measurements, which is already somewhat limited. On the other hand, tripling the thickness of the suspension reduces the solarization effect and prolongs the time of each observation.

RESULTS

The course of gas exchange in *Chlorella vulgaris* under very high light intensities is described by Fig. 1. (Unless otherwise indicated, all data apply to measurements made on *Chlorella vulgaris* in buffer No. 9.) Oxygen evolution or uptake, indicated as millimeters of pressure of manometric fluid, is plotted against time of illumination. The curves have been shifted vertically so that their 5 minute readings coincide. (The error

involved in the 5 minute, and at this very high intensity probably also in the 10 minute, readings has been already pointed out.) At these intensities the initial rate of oxygen evolution soon falls off. There follows an oxygen uptake which increases to, and for some time remains at, a fairly constant rate. A comparison shows that this constant rate of oxygen uptake is much greater than the rate of oxygen uptake indicated by the curve for dark respiration which has been included.

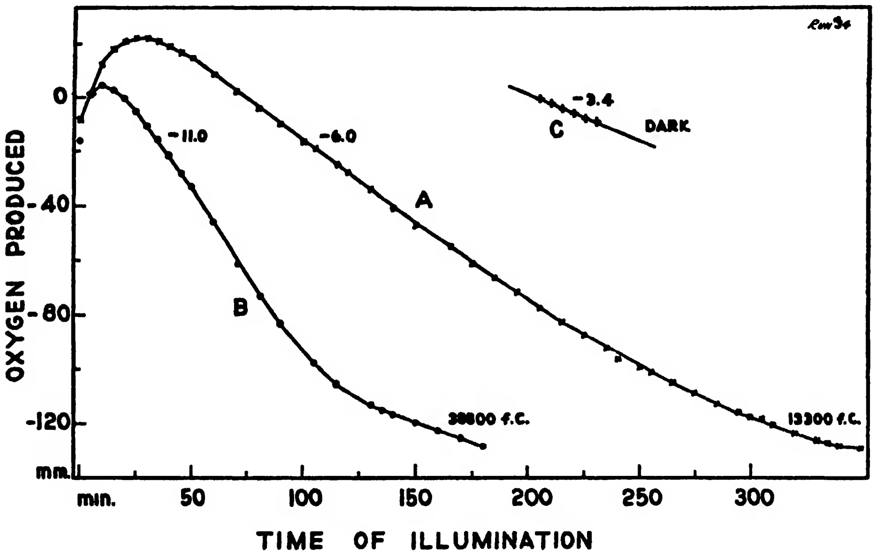

FIG. 1. Course of gas exchange in *Chlorella vulgaris* exposed to 13,300 f.-c. and 38,800 f.-c. of light. Oxygen produced or consumed is expressed in millimeters of pressure on the manometer. The rate above each curve is millimeters per 10 minutes.

After the consumption of a certain volume of oxygen (which occurs earlier at higher intensities) the slope (rate) begins to decrease. Runs carried out for even longer periods than those illustrated here have shown that the rate continues to decrease approaching zero. At about the time the die-away begins there is observable to the eye a distinct bleaching of the cells which continues until they are completely colorless, both microscopically and macroscopically.

It should be noted that the effects shown in Fig. 1 cannot be associated with a change in the CO_2 concentration of the buffer. A change of 100 mm. manometer pressure (56 c.mm. O_2) causes a change of only about 3.4 per cent in the CO_2 concentration provided by buffer No. 9. The highest rate ever noted in this buffer (35 mm./10 minutes, Fig. 10) was maintained for 70 minutes without any evidence of inhibition.

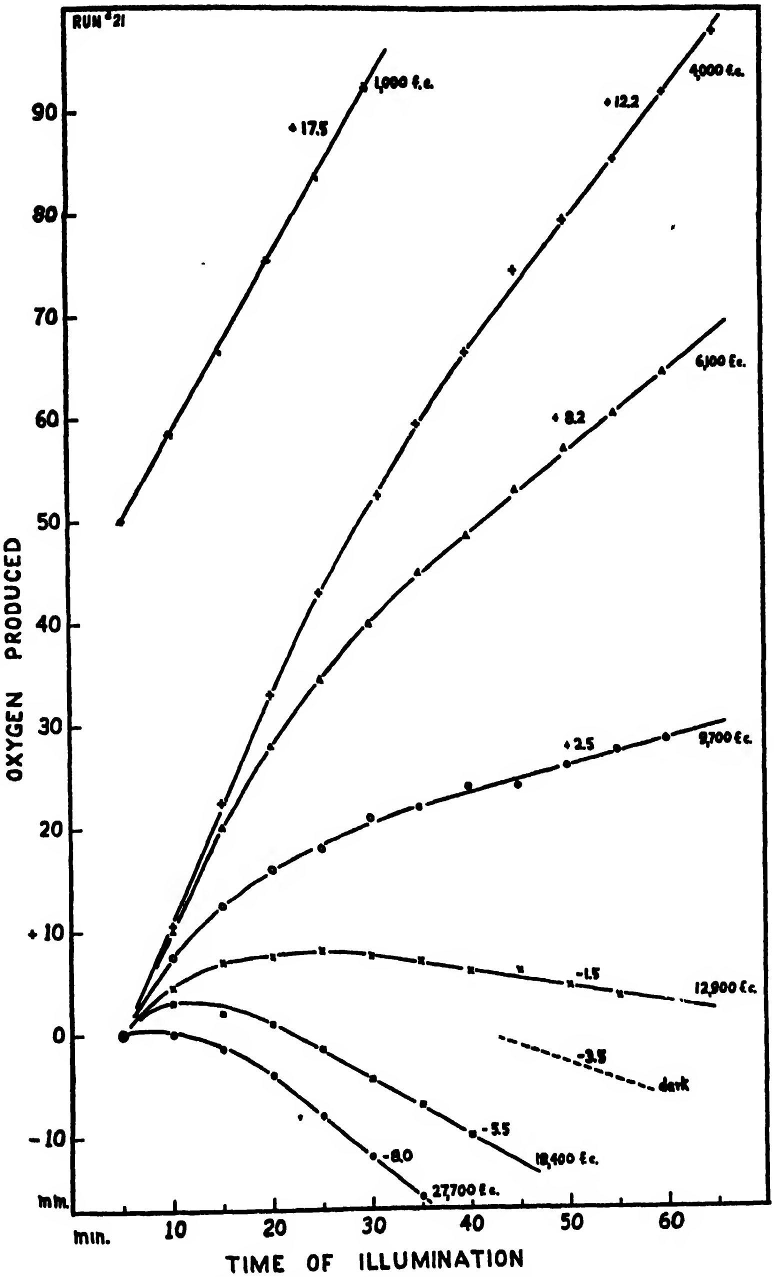

FIG. 2. Final steady rates of oxygen exchange depend on the incident light intensity. The rates are expressed as millimeters pressure change per 10 minutes and are shown by the numbers just above each curve.

In Fig. 2 is a family of curves for different light intensities, each obtained on a separate aliquot portion of a single batch of cells. At lower light intensities the final rate remains positive showing only partial inhibition of photosynthesis. When curves like those of Fig. 2 are run at still lower intensities a point is reached at which no decrease in rate can be observed. In fact at these lower intensities (in this case 1,000 f.-c. or less) there is often a slight increase in rate during a long run. It is of interest that the initial rates at 4,000 and 6,100 f.-c. are nearly identical and some-

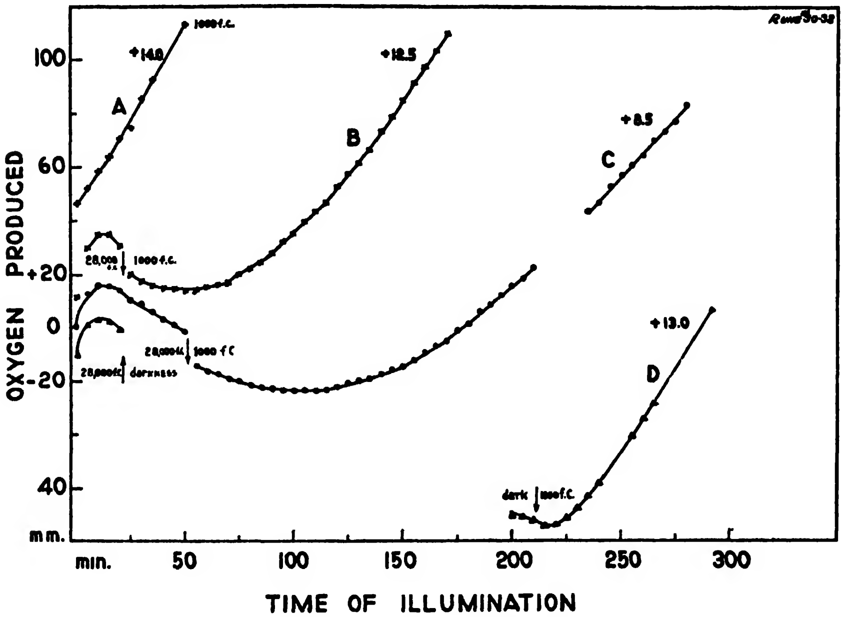

FIG. 3. Progressive injury of cells by 28,000 f.-c. of light and recovery in darkness (D) and in 1,000 f.-c. (B and C).

what higher than the rate at 1,000 f.-c. At some intensity between 1,000 and 4,000 f.-c. light saturation is reached and injury becomes apparent only after a considerable period of illumination.

Recovery experiments indicate that, at least at the higher intensities, there is an injury which becomes more severe with time. Several such experiments are illustrated in Fig. 3. Curve A shows photosynthesis in 1,000 f.-c., an intensity somewhat below the so called light saturation and at which no depression in rate has ever been observed for these cells. Curves B and C show the recovery of cells exposed to 28,000 f.-c. for 20 and 50 minutes respectively, and then changed to 1,000 f.-c. intensity. Curve D shows the recovery of cells exposed for 20 minutes to 28,000 f.-c.,

given a 175 minute rest in darkness, and then exposed to 1,000 f.-c. The numbers adjacent to the curves are rates of oxygen evolution, expressed

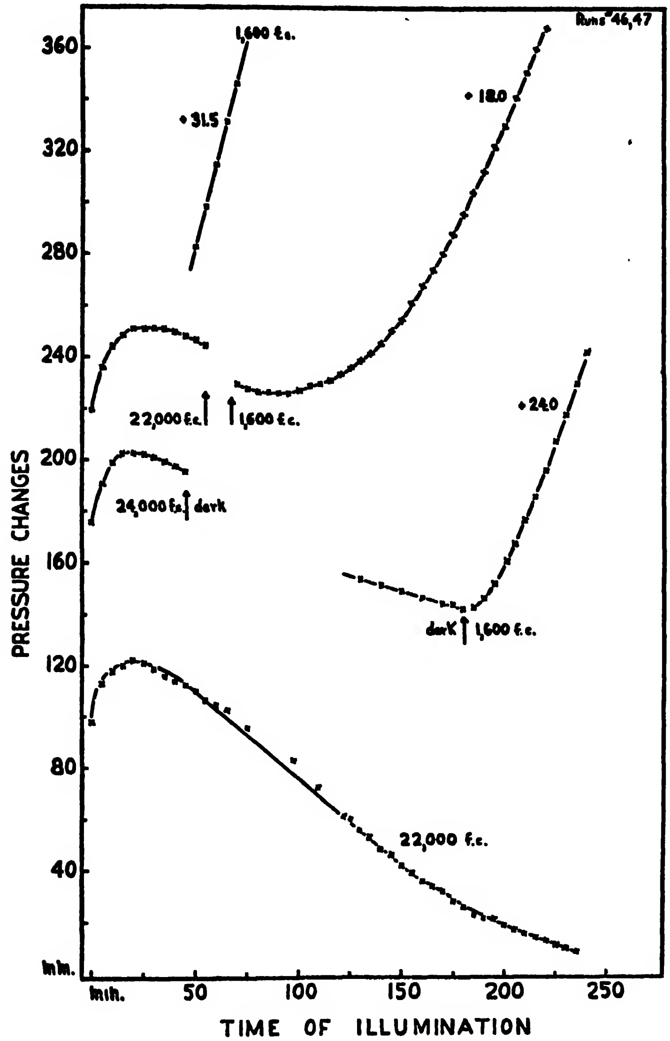

FIG. 4. Recovery curves of *Chlorella* in nutrient solution saturated with 5 per cent CO_2.

as increase in millimeter pressure per 10 minutes, after the curve has become a straight line.

It is apparent that on the constant downward slope of a curve such as either of those of Fig. 1 a progressive injury is taking place. As compared

to B (Fig. 3), recovery after the long exposure of C is much slower and less complete (though a still longer recovery time might have resulted in a further increase in rate). Curve D, as compared with B, indicates that recovery takes place in darkness as well as in 1,000 f.-c. But if exposure to strong light is prolonged as in either of the curves of Fig. 1, no recovery can be demonstrated, even after 12 hours of darkness.

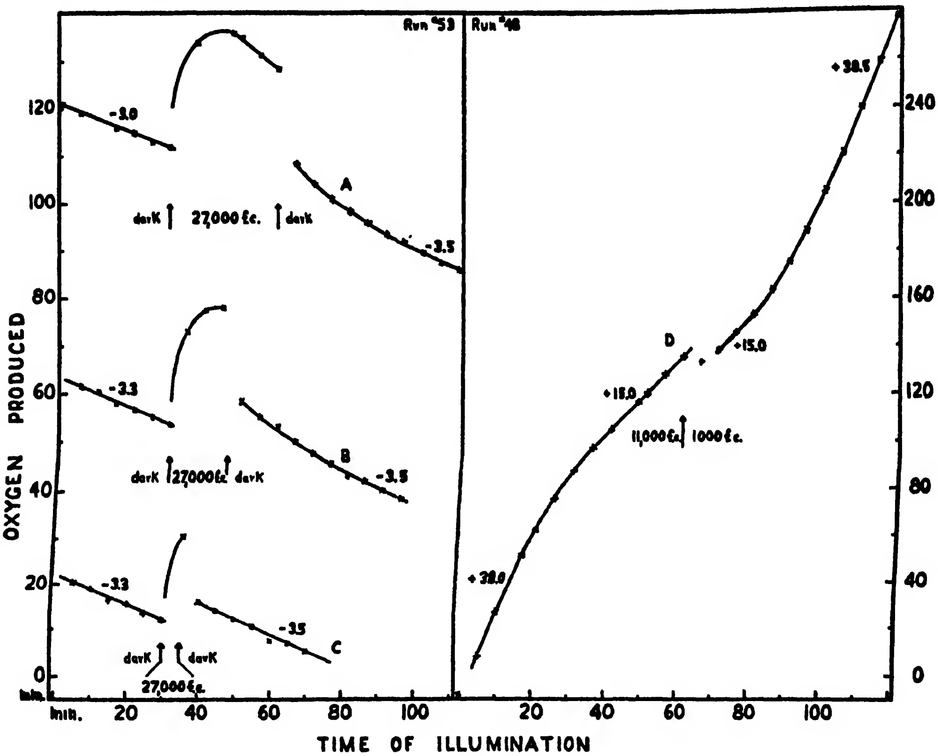

FIG. 5. Recovery in darkness shows stimulated respiration after exposure to bright light (A, B, C). There is complete and rapid recovery under 1,000 f.-c. (D).

The recovery experiments described above as performed in K_2CO_3-$KHCO_3$ buffer have been repeated using nutrient solution saturated with 5 per cent CO_2. Several of the curves thus obtained are shown in Fig. 4. The results are essentially similar.

It, therefore, seems reasonable to suppose that along the constant downward slope of the curves such as those of Fig. 1, there occurs with time a progressive injury which becomes irreversible when the die-away is reached.

Two other types of recovery experiments are illustrated in Fig. 5. Curves A, B, and C demonstrate the return to normal respiration in the dark after

30, 15, and 5 minutes exposure, respectively, to 27,000 f.-c. intensity. It is seen that there is a carry-over of the increased oxygen uptake after the light is turned off. And the return toward the normal rate of respiration is slower after longer exposure to the intense light.

Curve D of Fig. 5 shows recovery in 1000 f.-c. after exposure to 11,000 f.-c. 10 minutes after the reduction in intensity the rate is identical with that obtained under 11,000 f.-c. Recovery then proceeds rapidly.

In Fig. 6 there is presented evidence as to the time relations involved in the process producing injury under very high light intensity. If instead of 1,000 or 1,600 f.-c. illumination during recovery, an intermediate intensity is used (in this case 7,500 f.-c.), there takes place a rather rapid readjustment to a new constant rate. And as seen in this family of curves, the final rate depends upon the time of previous exposure to 23,000 f.-c. The final rate is thus a measure of the combined effects of 7,500 f.-c. and the preliminary exposure to 23,000 f.-c. If there is made the simplifying assumption that after 35 minutes exposure to 23,000 f.-c. the cells exhibit no photosynthesis (rate of O_2 exchange $= -6.0$ under 7,500 f.-c.) and further that this level of oxygen uptake prevails for all other curves, then the addition of 6.0 to the final rate for each curve will give the "true rate of photosynthesis." The rates of photosynthesis so obtained are plotted in Fig. 7 against time of previous exposure to 23,000 f.-c. (figures in parentheses at the end of each curve of Fig. 6). The shape of this curve is, of course, independent of our assumption that a zero photosynthesis is reached. A very similar curve obtains if the approximate initial rates under 7,500 f.-c. are plotted instead of final rates. Obviously a very great reduction in photosynthesis takes place within the first few minutes exposure to 23,000 f.-c. (in this case 50 per cent inhibition in 4 minutes).

It has been found useful to examine the constant slopes (see Fig. 1) as a function of light intensity. Occasionally there occurs a slight break in the constant slope. (This takes place at the 50 minute point in the curve of Fig. 1 for 38,000 f.-c. which was especially selected as a demonstration.) Such a break has shown up but rarely, although most of the curves have been followed only for about 50–70 minutes. For consistency, then, in the following discussion reference will be made only to the first constant slope, which will be called the "final rate of oxygen evolution" or simply the "final rate."

A number of families of curves similar to that of Fig. 2 have been obtained for cells grown under various conditions. Cells were grown in darkness and in light, in 1 per cent and 0 per cent glucose, with air and with 5 per cent CO_2 in air bubbled through. There are six different combinations

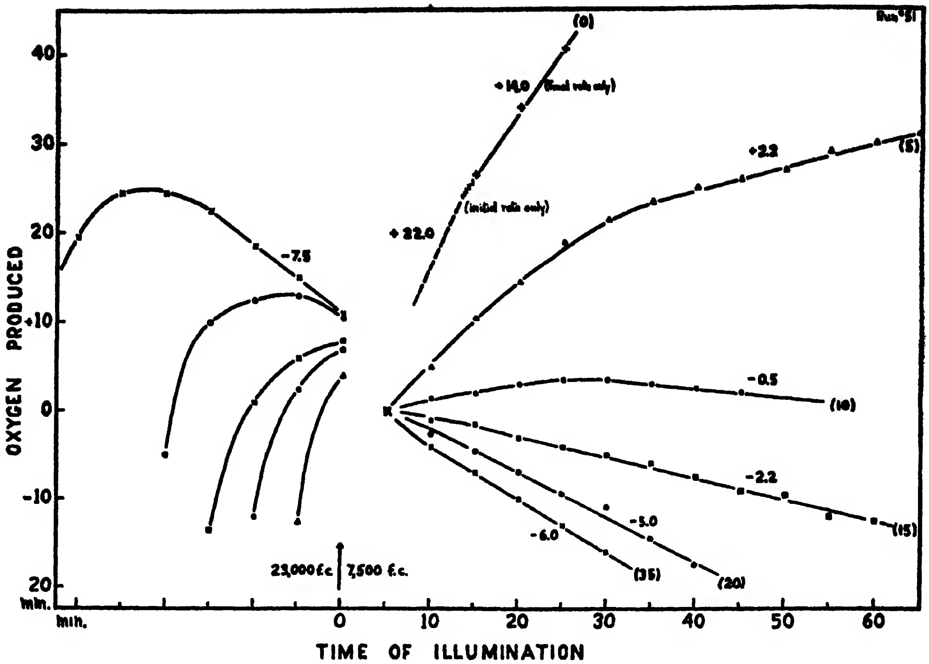

FIG. 6. Readjustment under 7,500 f.-c. after exposures of 5 to 35 minutes to 23,000 f.-c. The time of exposure to the intense light is given at the end of each curve (top curve no exposure, bottom curve 35 minutes before turning on 7,500 f.-c.).

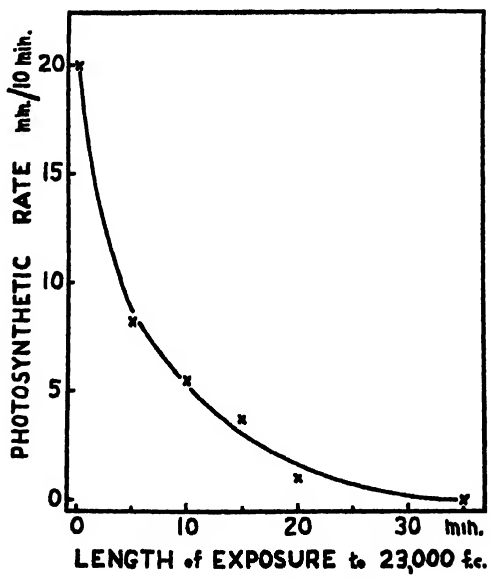

FIG. 7. Residual photosynthetic activity after varying exposures to 23,000 f.-c. A rate of 20 equals 100 per cent for this batch of cells under 7,500 f.-c.

of these factors under which algae can be grown. In Figs. 8, 9, and 10 the final rates obtained from each family of curves are presented as a function of light intensity. Final rates at higher intensities were determined as indicated in Fig. 2. Points at lower intensities were determined by shorter runs. Rates at these lower intensities are plotted in the insets with intensity on an expanded scale.

Inspection of the six curves reveals four points of interest:

1. Above a certain critical light intensity, which depends upon the previous history of the cells, the rate of oxygen evolution falls off in a continuous and predictable manner with increasing light intensity, finally becoming negative.

2. Cells grown in darkness show a depression in rate of oxygen evolution at lower intensities than comparable cells grown in light; *i.e.*, they are more sensitive to light.

3. Cells grown in high CO_2 in light show a depression in rate of oxygen evolution only at much higher light intensities than comparable cells grown in low CO_2; *i.e.*, they are less sensitive to light. This is exhibited in the curves as a broad plateau at which oxygen evolution is independent of light intensity.

4. Regardless of previous history all curves approach a maximum rate of oxygen uptake at high light intensities. This rate is about two to four times as great as the rate of oxygen uptake in dark respiration before exposure.

The data of Fig. 11 (as well as other data not here illustrated) obtained with *Protococcus* demonstrate that the qualitative nature of the effect described for our strain of *Chlorella vulgaris* is not a species peculiarity. Similar data have also been obtained for *Chlorella pyrenoidosa.*

The plateau effect illustrated in Figs. 9 and 10 has been studied further in the experiment shown in Fig. 12. Cells grown in 5 per cent CO_2 and light were studied at high intensities, using both buffer No. 9 and also buffer No. 11 which has over 3½ times the CO_2 concentration of No. 9. The dotted portions of the curves for the final rates are plotted with some uncertainty. Even so, the curves are of importance in two respects. It is demonstrated that the solarization effect cannot be due simply to inadequate CO_2 provision. (This point is also borne out by the experiments of Fig. 4 where nutrient solution saturated with 5 per cent CO_2 was used.) At intensities greater than 12,000 f.-c., cells receiving 3½ times the CO_2 concentration show very little increase in the final rate. On the other hand, there is a considerable increase in rate in the plateau region.

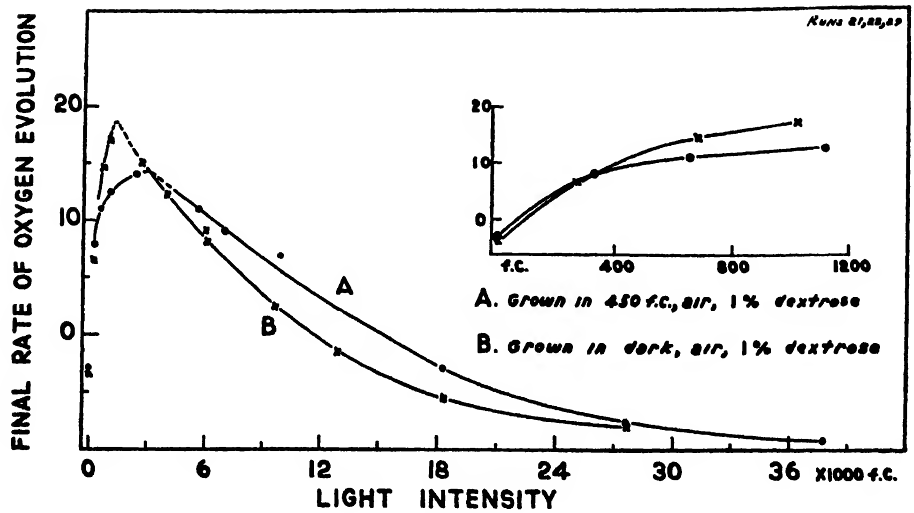

FIG. 8

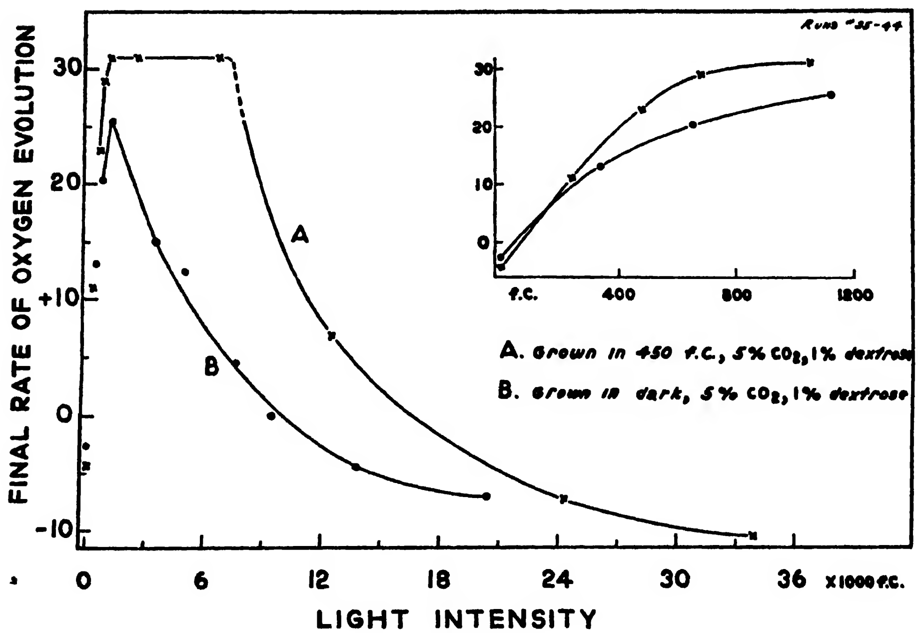

FIG. 9

FIGS. 8, 9, and 10. Showing the effect of conditions of culture on response to light

It would be highly desirable to locate within the cell those mechanisms by which the above effects might be brought about. The use of HCN and of narcotics such as the urethanes might be expected to throw some light on this question. Data on the influence of narcotics have not as yet been obtained. A few experiments have been carried out on the influence of HCN. One of these is illustrated in Fig. 13. It was found by preliminary

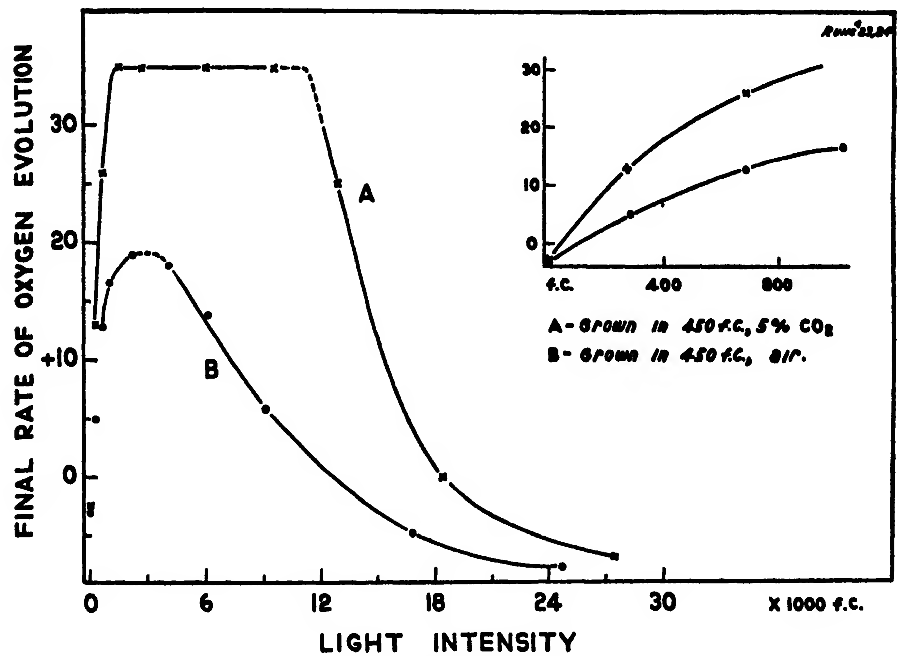

Fig. 10

experiments that for cells grown in light with 5 per cent CO_2, a 0.01 M KCN solution in the buffer mixture would prevent any oxygen evolution above the compensation point in the intensity range of about 200 to 1,000 f.-c. This is a somewhat higher concentration than has commonly been used in the study of photosynthesis. Warburg's (1920) data in one of his experiments indicate that at about 1,800 f.-c. he was approaching a compensation point with 0.005 M KCN. A lower concentration such as 0.005 M KCN still allows for our cells an appreciable oxygen evolution in this intensity range. Both of these concentrations stimulate the rate of dark respiration.

In Fig. 13 the upper curve was interpolated from the five experimental points by analogy to the upper curve of Fig. 10 for cells grown under identi-

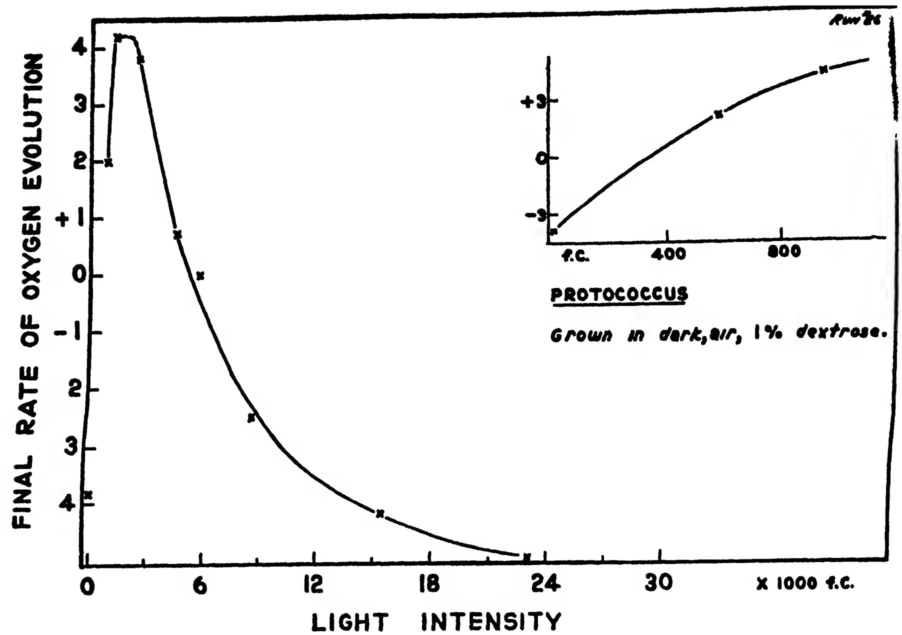

Fig. 11. Rate-intensity curve for *Protococcus*, illustrating qualitative similarity to *Chlorella.*

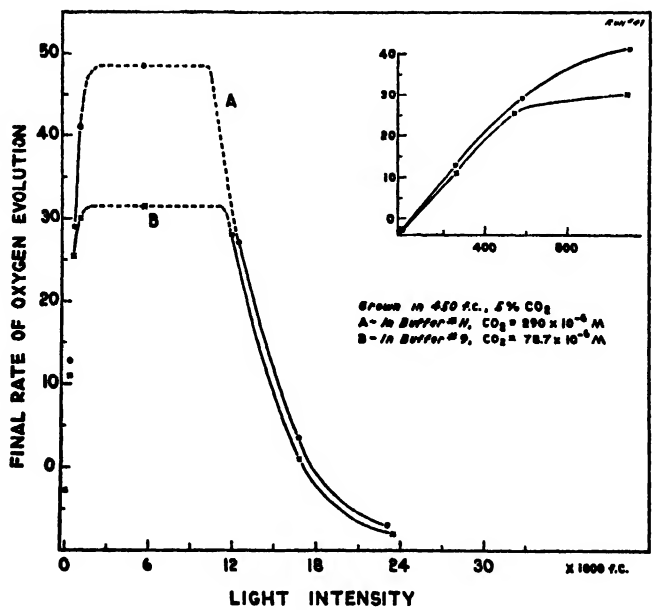

Fig. 12. Effect of CO_2 tension on the light response. High CO_2 (A) increases the maximum rate but does not prevent injury at high intensities.

cal conditions. The lower curve of Fig. 13 was obtained when 0.01 M KCN was added to the buffer mixture. Both curves seem to be approaching the same limiting value, though there are too few points to establish this with certainty. Evidently a cyanide concentration which will prevent any net photosynthesis as measured by oxygen evolution does not block the mechanism responsible for the oxygen uptake at high intensities.

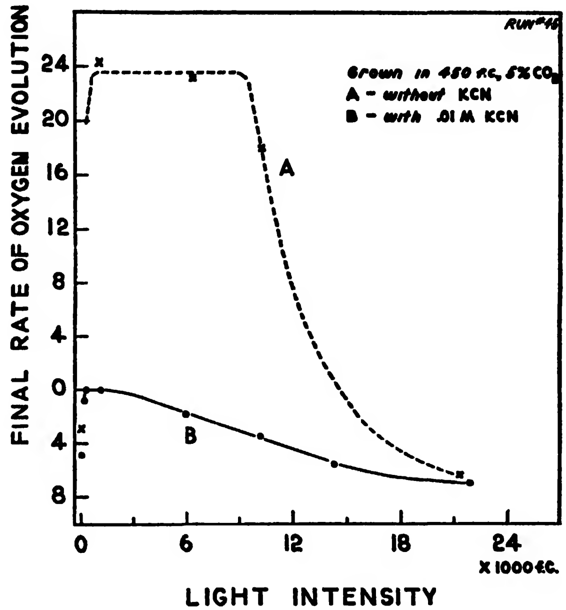

FIG. 13. Effect of KCN on response to light

DISCUSSION

It is rather surprising that the phenomena noted here have not already been adequately described. Probably no previous workers have used so great a range of light intensity. However, a number have used intensities ranging up to about 10,000 f.-c. And Smith (1937) has described certain of the kinetic properties of photosynthesis on the basis of observations made at intensities up to 282,000 lux using the water plant *Cabomba*. At the higher intensities he noted a "small decrease in rate to take place after three or four hours." He obviated this by using a red filter which probably also cut the total intensity in half.

Only two previous papers give results directly comparable with ours. Emerson (1935), using a similar technique, found an injurious effect of 4 or 16 hours exposure to 4,500 f.-c. on *Chlorella*, but only when the CO_2 supply was either inadequate or lacking. Injury was noted by a considerable decrease in the photosynthetic rate after as compared to the rate before exposure. He did not consider the accompanying chlorophyll destruction great enough to account for the decline in photosynthesis. For instance, a 70 per cent decrease in rate was accompanied by only a 20 per cent loss of chlorophyll.

This effect found by Emerson is suggestive of, but not directly com-

parable with, our experience that cells grown in low CO_2 (0.03 per cent) are more susceptible to a depressing effect of light on the rate of oxygen evolution. We are probably dealing in different ways with the same phenomenon. However, in the same paper[5] is the statement: "Never in the course of hundreds of experiments has the writer found a decline in rate attributable to high light intensities, though this effect has often been looked for, and light intensities up to about 100,000 meter candles have been used." If the algae were grown either in bubbling 5 per cent CO_2 as in his earlier work (1929), or in the 6.7 per cent CO_2 used in this experiment, then his cells were probably comparable to those described by the upper curve of Fig. 10; *i.e.*, more resistant to light injury.

A second paper offering direct comparison is that of Fockler (1938) in which he described the effect of high light intensity on the shade fern *Trichomanes radicans*. His light intensities (expressed by him only in relative units) probably were not as high as ours. But his use of a thin-leaf plant (fronds only about 1 cell thick) should have reduced shading of cells to a minimum.

Fockler is not explicit about his experimental method. He apparently measured by the Winkler method the oxygen dissolved in the water circulated over the submerged frond. He does not refer to Emerson's (1935) paper and may not have provided adequate CO_2. His rates were measured only at hourly intervals. However, after 1 hour's exposure to sunlight he got no apparent photosynthesis but an oxygen uptake which increased in rate until the 3rd hour. Fronds exposed for 1 or 2 hours showed partial recovery of normal photosynthetic activity in 5 days, full recovery in 14 days of moderate light. Fronds exposed for 4 hours had not yet fully recovered in 14 days. A similar though less pronounced effect was obtained with *Laminaria digitata*. Fockler was interested in the effect of light on respiration. Much of his data are on colorless plant tissue. On such material he noted at an hour's exposure an increase in respiration up to 100 per cent. With longer illumination this gradually fell off, approaching its original value. Because of this he believes that his experiments with *Trichomanes* show the result of two processes: an increased respiration and an inactivation of photosynthesis, both of which probably result from "eine Störung im kolloidalen System des Protoplasmas."[6]

The recent work of Stålfelt (1939) (which has come to our attention since the experimental work was done) seems also to have some bearing

[5] Emerson, R., Cold Spring Harbor symposia on quantitative biology, Cold Spring Harbor, Long Island Biological Association, 1935, **3**, 130.

[6] Fockler, H., *Jahrb. wissensch. Bot.*, 1938, **87**, 89.

on the problem. Of particular interest are his experiments on two species of lichens. In one experiment over a period of 6 days (10 hours light of 16,000 lux, 14 hours dark) there occurred daily a light-inhibition of photosynthesis which amounted to about 26 per cent of the average rate. In each 14 hour period recovery was about 23 per cent complete. This reversible light-inhibition was further shown to be proportional to light intensity from 4,000 to 48,000 lux and independent of temperature up to 20°C., the optimum temperature for photosynthesis in these plants. Above 20° an additional temperature-inhibition took place which was not reversible in darkness. In fact an inhibition of photosynthesis could be obtained by exposure to higher temperatures (20–28°) in the dark.

Stålfelt's work indicates that the light-inhibition occurring in lichens is probably closely related to the similar effects reported here for *Chlorella* and suggests that the phenomena observed for *Chlorella* may profitably be studied also as a function of temperature. But his observations are of no immediate aid in the interpretation of our data.

The authors realize full well the difficulties involved in reaching a full explanation of the observed phenomena. The inadequacy of our data is paralleled by the lack of complete or directly comparable data in the literature. The task of this discussion is, therefore, to arrive at some working hypothesis which will account for the data so far obtained.

It should be emphasized that all the data apply merely to the uptake or evolution of oxygen by the algal cells. Nothing at all is known about the total CO_2-O_2 exchange.

The evidence from our data is of two kinds, that from the "time" curves (Figs. 1–7) and that from the "intensity" curves (Figs. 8–13).

In very high light intensities the rate of oxygen uptake is much greater than the rate of dark respiration. For want of a better name, this excess oxygen uptake is called "photo-oxidation," at the same time recognizing that it may not be the simple photochemical reaction which the name implies. That photo-oxidations sensitized by chlorophyll can take place has been shown by the work of Gaffron (1933) and of Kautsky and Hormuth (1937).

The problem, then, is to relate the three processes of respiration, photosynthesis, and photo-oxidation in such a way as to account for the observed O_2 exchange. In introducing the term photo-oxidation it is assumed that respiration is constant and independent of light. The O_2 exchange now depends on the balance between photosynthesis and photo-oxidation. In regard to the photosynthesis two alternate hypotheses are suggested: (1) it

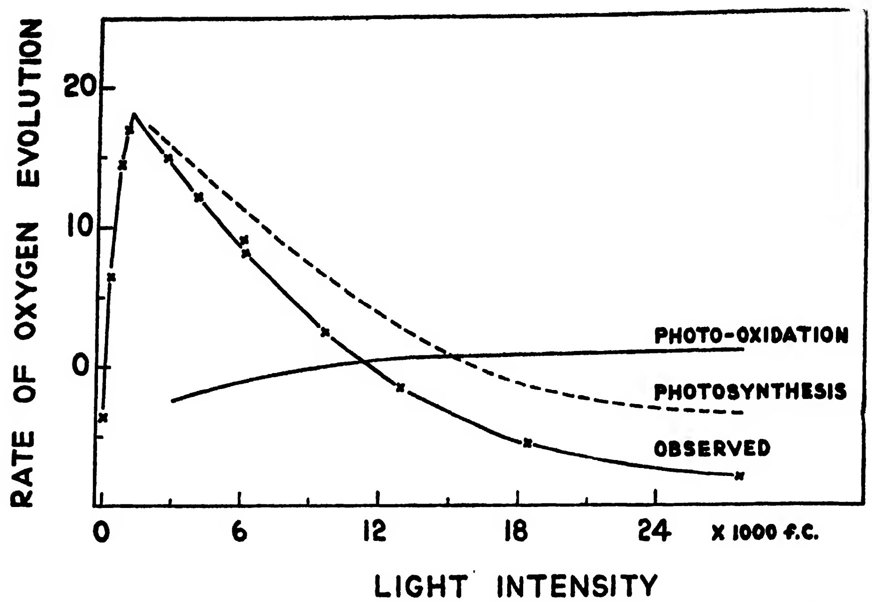

FIG. 14. Hypothetical intensity curve for photo-oxidation. Photosynthesis inhibited.

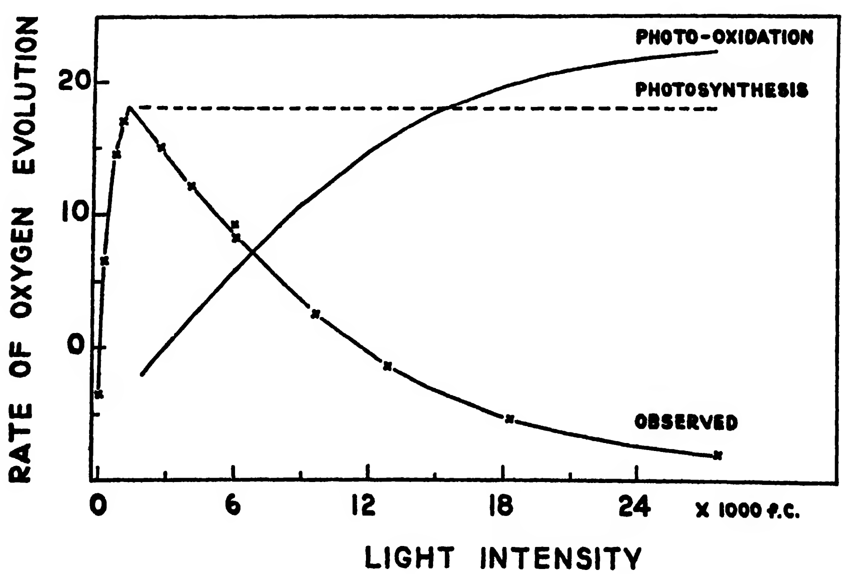

FIG. 15. Hypothetical intensity curve for photo-oxidation. Photosynthesis constant at high intensities.

is progressively inhibited by increasing light intensities; or (2) it continues at a maximum value at all higher intensities.

These two hypotheses are illustrated graphically in Figs. 14 and 15 based on the data obtained from the family of curves of Fig. 2 and curve B of Fig. 8.

In Fig. 14 the assumption is followed that zero photosynthesis is reached within the range of intensities studied. The "photosynthesis" curve is drawn quite arbitrarily except that it fulfills this condition. The observed final rate of O_2-evolution must be the net effect of photosynthesis and photo-oxidation (plus respiration). The hypothetical photo-oxidation curve is thus obtained by point-by-point subtraction of the "observed" from the photosynthesis curve. It, of course, represents a negative evolution of oxygen. Like the photosynthesis curve it is plotted with its base line at the level of dark respiration. Thus, at the intensity at which the curves of photosynthesis and "photo-oxidation" cross, the net O_2-evolution is the -3.5 (mm./10 min.) of dark respiration. As here drawn the photo-oxidation approaches a maximum value, although this is not at all certain since the photosynthesis curve has been drawn with a good deal of uncertainty.

Fig. 15 results from the second hypothesis that photosynthesis continues at a maximum value at all higher intensities. The maximum rate of photosynthesis is taken as the value approached at intensities of about 1,000 f.-c. (where photo-oxidation must be small).

An essential difference in the consequences of the two hypotheses is that the first requires a much smaller rate of photo-oxidation, approaching the magnitude of dark respiration. This is in accord with other observations in the literature on the effects of light on respiration. The second hypothesis, on the other hand, requires tremendously higher rates of photo-oxidation. We can find no evidence for such high rates. In fact, the complete lack of response to 1,000 f.-c. after 20 and 50 minutes in 28,000 f.-c. (curves B and C, Fig. 3) indicates that the photosynthetic mechanism is inactivated in both cases. The lack of response to a 3½ times increase in CO_2 concentration at high intensities (Fig. 12) also makes the assumption of a maximum photosynthesis at these high intensities highly unlikely. And it is impossible to account for the constant downward slopes (as those of Fig. 1) and the character of the recovery curves (Fig. 3) by the assumptions involved in the second hypothesis. However, the second hypothesis has been considered since it represents a contrast to the first.

Assuming, therefore, that photosynthesis is progressively inhibited by increasing intensities, the curves of Fig. 3 indicate that under 28,000 f.-c.

intensity the cells suffer a progressive injury with increasing exposure, from which they recover less completely and more slowly. However, the complete lack of response to 1,000 f.-c. after 20 and 50 minutes in 28,000 f.-c. *(B and C) seems to show that the photosynthetic mechanism is fully inactivated in both cases. This fact explains the constant rates of oxygen* uptake in Fig. 1 during a period in which progressive injury is taking place. There must be two distinct phenomena involved: (1) the complete inactivation of the photosynthetic mechanism within the first few (20–30) minutes, followed by (2) a progressive destruction of some cellular material which eventually goes to completion and stops photo-oxidation (the total O_2-uptake approaches a limit). When the second process has gone so far that the photo-oxidation rate begins to decrease, the cells are completely bleached and can no longer recover. It may well be, therefore, that photo-oxidation depends on chlorophyll absorption. But the inactivation of the photosynthetic mechanism is here due to another effect and there need be no direct relation between chlorophyll content and depression of photosynthesis. The maximum absorption of oxygen takes place when the photosynthetic mechanism has been inactivated and there is still much chlorophyll present.

Two characteristics of the phenomenon involved in the process of photosynthesis inactivation are described by Figs. 5 and 7. Fig. 7 indicates that the process is extremely rapid, and a comparison of Figs. 5 and 7 makes it clear that the 5 minutes of bright light which greatly depress photosynthesis do not appreciably affect the rate of oxygen uptake (as indicated by the "carry-over" into the dark).

Thus the rapid process required to reach the final steady rate at a given light intensity is considered a destruction of some factor in the photosynthetic mechanism. This factor is reduced to a concentration at which it is maintained at a steady state for any given intensity. The concentration of this factor would then limit the photosynthetic rate and, along with photo-oxidation, determine the final rate of oxygen evolution. Such a conception would apply equally well to all of the curves of Fig. 2. The maintenance of the steady downward slope of curves A and B of Fig. 1 is now easily explained. The progressive injury to photosynthesis does not affect the rate since photosynthesis has already been stopped by the destruction of some photosynthetic factor during the first few minutes of irradiation. The die-away is explained as a decrease in photo-oxidation when its substrate or sensitizer is almost used up.

At lower light intensities (4,000–12,000 f.-c., Fig. 2) only partial inhibition of photosynthesis takes place, and a steady but reduced rate is observed. This does not result in permanent injury during the time of ob-

servation. Recovery in 1,000 f.-c. from such a condition is rapid (see Fig. 5, curve D).

As already pointed out, the intensity curves in Figs. 8, 9, and 10 show that with increasing light intensities the final rate of O_2-uptake approaches a common limiting value apparently independent of the history of the cells. On the other hand, the maximum possible rate of photosynthesis varies greatly with the previous history of the cells. Again, this points to the first hypothesis that all photosynthesis has been stopped by the very high light intensities. Otherwise it would be necessary to assume that the same differences in previous history which favor high photosynthetic rates also favor proportionately high photo-oxidation rates.

Further mention should be made of the two types of intensity curves such as A and B of Fig. 10. In both cases measurements were made in the No. 9 buffer ($CO_2 = 78.7 \times 10^{-6}$ M). For curve A this is only about $\frac{1}{17}$ as great a CO_2 concentration as the 5 per cent CO_2 in which the cells were grown. Most measurements of intensity curves for algae have been made under similar conditions. For the cells of curve B the buffer provides CO_2 concentration seven times greater than that in which they were grown. In this case the rate of photosynthesis seems to be limited by some internal factor, probably the same one attacked during the first few minutes of exposure to high light intensities. In regard to curve A the suggestion is here proposed that cultures in high CO_2 and light develop a photosynthetic mechanism of high rate capacity. Buffer No. 9 in which the runs were made furnishes too little CO_2 for the cells to reach their maximum rates; and so over a range of several thousand foot-candles intensity CO_2 is the limiting factor. It is possible that through this range of increasing light intensity, inactivation of the photosynthetic mechanism has taken place to the same extent as seen in the lower curves (Figs. 9 B and 10 B). But the original capacity for photosynthesis is so great that CO_2 remains the limiting factor over a considerable range. This is supported by the results shown in Fig. 12. It seems likely that if the CO_2 concentration were increased enough, curve A would rise to a very high value. The plateau would then disappear and the curve would show the effects of partial inactivation of the photosynthetic mechanism at intensities much less than 12,000 f.-c.

If Fig. 14 be accepted as picturing the relation between photo-oxidation and photosynthesis which results in the observed curves, then in all cases the sharp downward breaks in the intensity curves are largely due to inactivation of the photosynthetic mechanism rather than to photo-oxidation which increases but slowly with increasing light intensity.

Unfortunately the data on cyanide inhibition are not complete enough

to be of much help. The points at 300 and 1,200 f.-c. (curve B, Fig. 13) and the data of Warburg (1920) seem to indicate that in this intensity range internal photosynthesis is not blocked. If this also holds for higher intensities, then interpretation is difficult since it is not certain to what extent the internal photosynthesis compensates for photo-oxidation. It is true that the cyanide curve (B, Fig. 13) looks like an inverted form of the hypothetical photo-oxidation curve of Fig. 14. However, lack of knowledge of the specific effect of cyanide at these higher intensities allows little weight to be placed on this similarity.

Obviously, more data must be assembled before the solarization effect of high light intensities can be completely explained. Some oxidation in excess of dark respiration takes place under very high light intensity. We have tentatively called this "photo-oxidation." We have examined two alternative hypotheses for the behavior of photosynthesis. The assumption of a maximum photosynthesis continuing under very high light intensities has been shown to be untenable. On the other hand, all of our data are consistent with the view that with increasing intensities photosynthesis is progressively inhibited while photo-oxidation is progressively increased at a much lower rate.

SUMMARY

1. The effect on oxygen evolution of *Chlorella vulgaris* produced by light intensities up to about 40,000 f.-c. has been studied by the use of the Warburg technique.

2. Above a certain critical intensity, which is determined by the previous history of the cells, the rate of oxygen evolution decreases from the maximum to another constant rate. This depression is at first a completely reversible effect.

3. With a sufficiently high intensity this constant rate represents an oxygen uptake greater than the rate of dark respiration. During such a constant rate of oxygen uptake a progressive injury to the photosynthetic mechanism takes place. After a given oxygen consumption the rate falls off, approaching zero, and the cells are irreversibly injured.

4. The constant rate of oxygen evolution (2 and 3) decreases in a continuous manner with increasing light intensities, approaching a value which is approximately constant for all lots of cells regardless of previous history.

5. Two alternative hypotheses have been presented to explain the observed phenomena. The more acceptable of these proposes quick inactivation of the photosynthetic mechanism, the extent of inhibition depending on the light intensity.

6. In *Chlorella vulgaris* solarization is influenced by the previous history of the cells.

LITERATURE CITED

Emerson, R., Cold Spring Harbor symposia on quantitative biology, Cold Spring Harbor, Long Island Biological Association, 1935, **3**, 128.

Emerson, R., *J. Gen. Physiol.*, 1929, **12**, 609.

Fockler, H., *Jahrb. wissensch. Bot.*, 1938, **87**, 45.

Gaffron, H., *Biochem. Z.*, Berlin, 1933, **264**, 251.

Holman, R., *Univ. California Pub.* (*Bot.*), 1930, **16**, 139.

Honert, T. H., van den, *Rec. trav. bot. néerl.*, 1930, **27**, 149.

Kautsky, H., and Hormuth, R., *Biochem. Z.*, Berlin, 1937, **291**, 285.

MacInnes, D. A., and Belcher, D., *J. Am. Chem. Soc.*, 1933, **55**, 2630.

Meier, F., *Smithson. Misc. Coll.*, 1934, **92**, No. 6.

Paauw, F. van der, *Rec. trav. bot. néerl.*, 1932, **29**, 497.

Smith, E., *J. Gen. Physiol.*, 1937, **20**, 807.

Stålfelt, M. G., *Planta*, 1939, **30**, 384.

Warburg, O., *Biochem. Z.*, Berlin, 1919, **100**, 230.

Warburg, O., *Biochem. Z.*, Berlin, 1920, **103**, 188.

THE EFFECTS OF VARIATIONS IN THE CONCENTRATION OF OXYGEN AND OF GLUCOSE ON DARK ADAPTATION

By R. A. McFARLAND and W. H. FORBES

(*From the Fatigue Laboratory, Harvard University, Boston*)

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INTRODUCTION

The functioning of the central nervous system appears to depend upon a continuous and adequate supply of oxygen and glucose. When the concentration of either of these substances in the blood is lowered to approximately one-half of its usual level, there is significant impairment in cerebral function. At one-third or one-fourth of the normal level, the individual lapses into coma. The effects of mild, intermediate, and severe degrees of anoxia on sensory and mental functions have been extensively studied. In the case of hypoglycemia however, only the relatively severe effects have received attention, mostly in connection with the treatment of diabetes and more recently in relation to the insulin-shock treatment of schizophrenia.

Since the general effects of anoxia on the central nervous system have been reviewed elsewhere (McFarland, 1932, 1937, 1939), only the changes relating to light sensitivity will be discussed here. Under moderate degrees of oxygen lack there is a general darkening and narrowing of the visual field and then a blurring of outlines or a decrease in visual acuity. With more severe anoxia, previous to loss of consciousness, there may be an intermittent cessation of all visual experiences. Pilots often report a general darkening of the visual field while flying at great heights (18,000 feet and above). In Douglas bag experiments in the laboratory, one is subjectively aware of the dimming of lights on being suddenly exposed to partial pressures of oxygen simulating 12,000 to 14,000 feet. If one is suddenly changed back to room air, there is a marked increase in the brightness of the lights. Controlled studies of light sensitivity appear to offer one of the most sensitive tests available of the initial as well as the advanced effects of anoxia. In one study, for example, the effects were first observed at 15.8 per cent O_2 or 7,400 feet altitude. The thresholds of light sensitivity were progressively raised with increasing deprivation of oxygen in both the rod and cone portions of the dark adaptation curve (McFarland and Evans, 1939).

The studies of hypoglycemia have not been so extensive as those of anoxia. Although several controlled experiments have been made, the observations for the most part are scattered and often incidental to other interests. After prolonged exercise on a bicycle ergometer while on a fat diet, Christensen *et al.* (1934) reported subjective visual phenomena and mental dullness which disappeared within 10 minutes after the ingestion of glucose. In a study of fasting in human subjects, Glaze (1928) found a decrease in their performance in mental tests, although no attempt was made to correlate the level of blood sugar with specific mental functions. The speculations of Barcroft (1938) relative to the blood sugar level and mental performance are suggestive but, as yet, have not been confirmed by experimentation. Evidence of a relationship between blood sugar level and brain function was proposed by Hoagland *et al.* (1937) in studies of the electroencephalogram during insulin hypoglycemia. They observed a decline in alpha wave frequency (Berger rhythm) of some 40 per cent which roughly paralleled the declining blood sugar curve. The effects of small injections of insulin (0.02 of a unit) on rats while learning to run mazes have given conflicting results. These variations in performance may be due to the influence of motivation on learning and conditioning. It has been demonstrated, for example, that small doses of insulin produce hypertonus and hypermotility of the stomach which may form the basis of variations in the "hunger drive" in the animals (Bulatao and Carlson, 1924; also Quigley, Johnson, and Solomon, 1929).

The similarity between the syndrome of anoxia and that of hypoglycemia, as far as the central nervous system is concerned, has been discussed by Olmsted and Logan (1923) and more recently by Gellhorn (1938). Olmsted and Taylor (1924) expressed the opinion that the fall in the sugar content of the blood causes depression of the oxidative processes in the brain cells to such a degree that the effects are similar to those of anoxia. Some basis for this view was obtained by Holmes (1930) and Wortis (1935). They found that the oxygen consumption of sliced brain tissue was roughly proportional to the concentration of dextrose in the solution and that the oxygen consumption of the brain tissue obtained from animals following the injection of insulin was lower than that of brain tissue obtained from animals used as controls. The application of these findings to the intact organism was made by Dameshek, Myerson, and Stephenson (1935), who found in human subjects that severe hypoglycemia decreased the difference in oxygen content between the arterial blood in the carotid and the venous blood in the internal jugular. They found at the same time that the blood flow appeared to be slightly decreased, but the method they used for meas-

uring the velocity of the flow has since been abandoned by its author. Similar results have been obtained by Himwich *et al.* (1937, 1939) and by Wortis and Goldfarb (1940) on schizophrenic patients. These studies suggest a decreased oxygen consumption in the brain during hypoglycemia but before this can be proved it must be shown conclusively that the amount of blood passing through the brain is not increased. Loman and Myerson's (1936) findings that the velocity of blood flow is decreased in severe hypoglycemia suggest strongly but do not prove that the *amount* also is decreased.

The brain is apparently less able to burn fats and proteins than other parts of the body. Glucose appears to be the main metabolic substrate. Even though (or perhaps because) the brain is composed largely of lipoid matter, fats probably are not oxidized (Jowett and Quastel, 1935). In the advanced stages of diabetes, when the rest of the organism obtains most of its energy from the oxidation of fat, the brain continues to oxidize carbohydrates (Himwich and Nahum, 1932). The cerebral supply of reserve carbohydrate in the form of glycogen is very limited (Kerr and Ghantus, 1936). The brain is, therefore, largely dependent on the sugar of the blood to satisfy its demands. In the light of these findings, a reduction in blood sugar would be expected to reduce the oxidation in the brain.

It is a reasonable hypothesis therefore that both anoxemia and hypoglycemia produce their effects in essentially the same way; namely, by slowing the oxidative processes. If this is the case, the effects should be upon the same parts of the organism and, in general, similar (though not necessarily identical, for one need not assume identical oxidative processes in all parts). Broadly speaking, the effects are similar. They are first observed in the nervous system in both cases, and their similarities are numerous. If in both cases it is reduced oxidation, rather than anoxia or hypoglycemia in themselves, which impair the mental functions, one would expect that in accordance with the law of mass action, oxidation would be increased by giving glucose in anoxia or oxygen in hypoglycemia. In either case, the organism should return toward normal, and this our experiments were designed to test. We lowered the blood sugar by injecting insulin and then increased the oxygen tension. On other occasions we exposed the subjects to low oxygen and increased the blood sugar. Finally, in a limited number of experiments, we lowered the concentrations of both of these substances simultaneously.

Other experimenters have used changes in blood pressure as criteria for analyzing the relation between hypoglycemia and anoxia in their action on the nervous system (Gellhorn, 1938). While this procedure is satis-

factory from the point of view of its objectivity, it is somewhat unsatisfactory in so far as a rise in blood pressure cannot be said to indicate either better or worse function on the part of the nervous system. In the experiments described below, we have used the thresholds for light sensitivity as criteria of the effects of hypoglycemia and anoxia on the central nervous system, believing that decreased sensitivity indicates poorer function. The phenomenon of the darkening of the visual field mentioned previously was followed by the usual procedure of studying night blindness; *i.e.*, the measurement of the return of the capacity to see in the dark following exposure to a light of high intensity. This test proved to be reasonably objective and reliable for use with human subjects and could be carried out satisfactorily even under moderately severe anoxemia or hypoglycemia.

Apparatus

The measurements of light sensitivity were made with a Hecht adaptometer which has been described in detail elsewhere (Hecht and Shlaer, 1938). The light adapting field, occupying about 35° visual angle and bright enough to show both cone and rod adaptation in the measurements (1,500 millilamberts) was exposed for 3 minutes. The test field, occupying 3° visual angle in diameter, was viewed 7° nasally with the right eye. In this region, the populations of rods and of cones are more nearly equal than in the center of the retina or farther in the periphery. The luminous fixation point used to keep the eye centered and steady was sufficiently removed from the measuring area so as not to interfere with its function. The measuring light was from the extreme violet end of the spectrum which furnished a clear color distinction between cone function and rod function. It was exposed in flashes of one-fifth of a second, long enough to produce good perception and short enough to be near the retinal action time.

Experimental Procedure

The test of light sensitivity consisted of two parts. First, the eye was exposed to the standard light adaptation. Then as the subject remained in the dark, the intensity threshold to flashes of violet light was determined approximately every 2 minutes until the end of the session. It is well known that dark adaptation proceeds in two stages, the first is very rapid and is over in a few minutes while the second is late in starting and continues for at least half an hour. The first part of the curve represents the behavior of the cones of the retina (color vision) and the delayed or slower second stage of adaptation the behavior of the rods (night vision) (Hecht, 1937). Each datum secured, therefore, represents the just perceptible light intensity after a certain time in the dark. Approximately fifteen such determinations give a curve of dark adaptation similar to those shown in Figs. 1 to 5. Time is plotted on the horizontal axis on an ordinary arithmetic scale and the logarithm of the intensity of a just perceptible light is plotted on the vertical axis.

The final thresholds in the various control curves represent a brightness of about one thousand-millionth of a lambert corresponding roughly to an illumination of one millionth of a foot-candle. Since the logarithms of such fractional numbers are negative and

inconvenient to use, the designers of the apparatus adopted a unit much smaller than the lambert; namely, the micromicrolambert ($\mu\mu l$) or 1×10^{-12} lamberts. All possible values of the threshold are thus given by positive logarithms.

All of our experiments were carried out in a chamber where the temperature (mean 70° F.) and ventilation were controlled with an air conditioning unit and where the concentrations of oxygen could be maintained at any desired level, the total barometric pressure remaining constant. Normal fasting subjects, varying in age from 25 to 37 years, were thoroughly practiced in the experimental procedure. A control dark adaptation curve was obtained before each experiment. Then the subject was exposed to low oxygen tensions by adding nitrogen to the atmosphere in the room from a cylinder of nitrogen attached to an outside manifold. Samples of air were obtained inside the chamber for the analysis of O_2 and CO_2 on the Haldane apparatus and samples of alveolar air were taken from the subject at the same time. After exposure to the low oxygen tensions for 20 to 30 minutes, another dark adaptation curve was plotted and then the subject was given glucose or oxygen as the case might be. In other experiments after the control curve was obtained, the subject was given insulin (5 to 8 units intramuscularly), tested again, and then subjected to varying oxygen tensions, and finally given glucose. During these experiments, samples of blood were obtained at various intervals for determination of the blood sugar (Folin micro method on unlaked blood from finger).[1] In certain experiments, as can be seen from the curves in Figs. 1 to 3, oxygen, nitrogen, or glucose was given while the eyes were still dark adapted and the effects observed without starting the whole procedure (exposure to light, etc.) over again.

The following series of experiments were carried out in accord with the technique and procedure described above.

I. Low oxygen series in 13.3, 11.4, and 10.0 per cent oxygen corresponding to altitudes of 12,000, 16,000, and 19,000 feet, respectively (6 subjects).

II. Control tests in air followed by tests during the inhalation of decreasing concentrations of oxygen going as low as 7.3 per cent oxygen in one case (5 subjects).

III. Low oxygen tests followed by the ingestion of 70 to 80 gm. of glucose (5 subjects).

IV. Insulin tests (5 to 8 units) followed by the inhalation of oxygen and the ingestion of glucose (9 subjects).

V. Combined effects of insulin and low oxygen (1 subject).

VI. Basal and non-basal series (10 subjects).

RESULTS

The results obtained in Series I in low oxygen are shown in Table I and Figs. 1 and 2. The average partial pressures of oxygen in the chamber and

[1] This method gives approximately the true glucose values and our figures therefore run 10 to 20 mg. lower than those obtained by methods which include the non-glucose reducing substances of the blood.

in the alveolar air are shown in Table II. The data obtained from one subject (W. F.) while inhaling a concentration of 9.45 per cent O_2 in the chamber are plotted in Fig. 1. Each discrete threshold, as determined by the procedure described above, falls quite close to the curve which has been

TABLE I

Thresholds of Light Sensitivity for Subject W. F. and the Mean for Six Subjects in Normal Air (20.94 Per Cent O_2) and While Inhaling Three Different Low Concentrations of Oxygen (Cf. Figs. 1 and 2)

Subject W. F.						Mean of six subjects				
Control (normal air)			9.45 per cent O_2 (20,000 ft.)			Time	Control (normal air)	13.4 per cent O_2 (11,500 ft.)	11.5 per cent O_2 (15,400 ft.)	10.1 per cent O_2 (18,500 ft.)
Time		Log *I*	Time		Log *I*		Log *I*	Log *I*	Log *I*	Log *I*
min.	*sec.*		*min.*	*sec.*		*min.*				
0	06	7.35	0	09	7.24	2	6.21	6.38	6.49	6.58
1	50	6.22	1	50	6.70	4	5.87	5.96	6.23	6.32
3	50	5.97	3	10	6.57	6	5.75	5.82	6.12	6.23
5	50	5.79	4	30	6.51	8	5.70	5.76	6.08	6.18
8	50	5.81	5	50	6.39	10	5.18	5.32	5.56	5.81
11	05	4.75	7	40	6.26	12	4.49	4.66	4.82	5.10
13	30	4.17	10	20	5.73	14	3.99	4.12	4.31	4.59
15	40	3.84	12	50	4.75	16	3.66	3.75	4.00	4.24
18	20	3.38	15	30	4.11	18	3.43	3.54	3.79	4.01
21	25	3.24	17	20	3.90	20	3.27	3.38	3.64	3.86
25	10	3.03	19	50	3.73	22	3.16	3.30	3.52	3.75
27	35	2.99	22	55	3.51	24	3.09	3.25	3.46	3.68
30	20	2.95	25	30	3.44	26	3.05	3.21	3.40	3.65
33	10	2.93	27	50	3.48	28	3.02	3.20	3.38	3.63
			30	00	3.55	30	3.01	3.20	3.37	3.62
			33	00	3.51	32	3.01	3.20	3.37	3.62
			After inhaling O_2 from a cylinder					After inhaling O_2 from a cylinder		
			33	50	3.20	33.3	3.00	3.12	3.21	3.29
			34	40	2.98	34.5	3.01	3.06	3.08	3.12
			35	55	2.92	36.0	2.98	3.01	2.95	2.96

Throughout the tables intensity (*I*) is in micromicrolamberts.

drawn through these various points. In comparing the curve in air with the curve in 9.45 per cent O_2 it appears that the cone portion was elevated by approximately 0.5 of a log unit and the rod portion by 0.6 of a log unit.

At the end of each test in low oxygen, the subject inhaled oxygen from a cylinder inside the chamber. Without exception, the final rod threshold fell within 2 or 3 minutes to about the level of the normal base line in air or even below it. Changes in the threshold were noticed after the subject

had been inhaling oxygen for less than one-half minute. This effect of excess O_2 is shown graphically in Fig. 1 for subject W. F. The data for the group of six subjects are shown in Table I.

The data for the mean curves of Fig. 2 were obtained while inhaling three different concentrations of O_2 in the chamber, namely 13.4, 11.5, and 10.1 per cent O_2. The experimental procedure was as follows. Each of six subjects served in the experiment on three different days, each time taking

TABLE II

The Average Tension of O_2 and CO_2 in the Chamber and in the Alveolar Air While Collecting the Data Shown in Table I and Figs. 1 and 2

	Control in normal air		1st Hour				2nd Hour			
	Alveolar air		Chamber air		Alveolar air		Chamber air		Alveolar air	
	pO_2	pCO_2	O_2	CO_2	pO_2	pCO_2	O_2	CO_2	pO_2	pCO_2
	mm. Hg	*mm. Hg*	*per cent*	*per cent*	*mm. Hg*	*mm. Hg*	*per cent*	*per cent*	*mm. Hg*	*mm. Hg*
Subject W. F. 9.45 per cent O_2 20,000 ft.	103.4	40.2	9.26	0.20	31.4	30.6	9.45	0.32	32.5	31.4
Mean six subjects 13.4 per cent O_2 11,500 ft. (*Cf.* curve I, Fig. 2)	102.7	39.4	13.44	0.18	52.7	34.1	13.38	0.28	51.6	35.3
Mean six subjects 11.5 per cent O_2 15,400 ft. (*Cf.* curve II, Fig. 2)	104.6	40.4	11.52	0.26	43.1	31.7	11.47	0.32	42.4	32.3
Mean six subjects 10.1 per cent O_2 18,500 ft. (*Cf.* curve III, Fig. 2)	102.5	39.6	10.15	0.21	34.5	29.4	10.13	0.29	33.7	30.1

the test in air followed by two tests in low oxygen, the first begun after approximately 20 minutes' exposure to anoxia, and the second after 1 hour. The control curve in air, therefore, is based upon the mean of eighteen experiments, and each of the three low oxygen curves upon the mean of six experiments carried out at intervals varying from 7 to 10 days. The low oxygen curves shown in Fig. 2 were those obtained during the 1st hour in the chamber. The results obtained during the 2nd hour, which will be discussed more fully below, were quite similar to the data shown in Table I and Fig. 2.

The method used in plotting the mean curves as shown in Fig. 2 was as

follows. The thresholds for each subject under the various conditions of oxygen deprivation were charted separately and from these individual

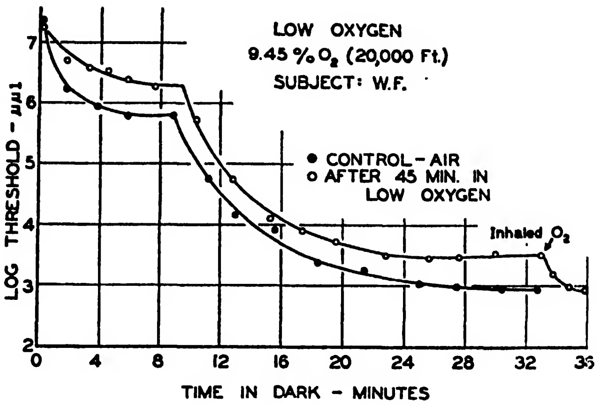

FIG. 1. Dark adaptation curves in air (solid circles) and in low oxygen (open circles) for subject W. F. Light sensitivity was reduced by lowering the O_2 tension. The threshold returned to normal upon inhalation of O_2 as indicated by the arrow at the end of 33 minutes. (*Cf.* Tables I and II.)

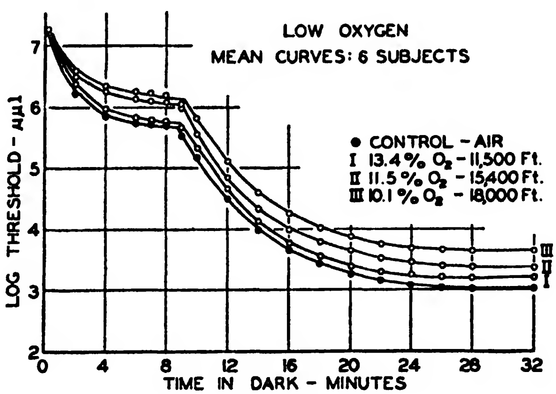

FIG. 2. Mean dark adaptation curves showing that if the oxygen tension was low, the thresholds were high. In the control curves each solid circle is based on the average of eighteen experiments; in the curves obtained at simulated high altitudes, each open circle is based upon the average of six experiments. (*Cf.* Tables I and II.)

curves, the deviation from the control was read off at constant intervals of 2, 3, or 4 minutes. In this way, it was possible to average the readings on a composite curve at similar time intervals following the light adapting stimulus. The composite curves give the appearance of the thresholds being more regular than was actually the case. The discrete points of each

observation did not deviate widely from the curve. This can be seen from the data in Fig. 1 for subject W. F. whose curve was quite typical of the others. The mean curves for six subjects in Fig. 2 show increasing anoxia gives an increasing elevation from the base line in both the cone and rod portions. The values of the final rod threshold for these six subjects ranged from 2.85 to 3.05 in the control series, from 3.05 to 3.22 in 13.4 per cent O_2, from 3.15 to 3.30 in 11.5 per cent O_2, and from 3.50 to 3.85 in 10.1 per cent O_2. In 10.1 per cent O_2 the inter-individual variability was therefore 0.35 of a log unit, or about twice what it was in less severe anoxia, indicating that at this high altitude, the differences between individuals were accentuated.

During each experiment, as stated above, three dark adaptation curves were plotted with the adaptometer; *i.e.*, one in air followed by two in low oxygen. The third test on the adaptometer was given to observe whether the effects were increased or decreased during the 2nd hour. In this way we believed that it might be possible to obtain some indication of the degree of acclimatization attained by each subject. The results were as follows. In 13.4 per cent O_2 both the cone and rod portions of the curve were approximately the same during the 1st and 2nd hours for all the subjects. There were no significant changes in the cone portions of the curve in 11.5 per cent O_2 but four of the six subjects were poorer in the rod portion by 0.10 to 0.25 of a log unit. In 10.1 per cent O_2 five of the six subjects were poorer in both the rod and cone sections of the curve varying from 0.15 to 0.40 of a log unit. During the final series in 10.1 per cent O_2 both the inter- and intra-individual variability in thresholds increased. The extent of the impairment and the variability were related to the extent of the changes in pulse rate and blood pressure and the decrease in partial pressure of oxygen in the alveolar air. In fact there appeared to be a fairly close relationship between the extent of the impairment in the final rod threshold of each subject and his ability to tolerate the effects of acute anoxia as judged by his general condition. Our observations are, therefore, in agreement with the suggestion of Clamann (1938) who believes that the extent of the impairment in light sensitivity under low oxygen might serve as a reliable objective test of one's ability to tolerate anoxia.

In Series II we analyzed the effects of anoxia on the dark adapted eye while the concentrations of oxygen were gradually decreasing. The results are shown in Table III and Fig. 3. After determining the lowest threshold for each subject in air over a period of 40 minutes (*cf.* Fig. 3), the air in the chamber was diluted with nitrogen so that within approximately 20 minutes the percentage of oxygen was reduced from 21 to 13, simulating

TABLE III

Thresholds of Light Sensitivity for Subjects W. F. and I. L. and the Mean for Five Subjects in Normal Air (20.94 Per Cent O_2) and in Decreasing Concentrations of Oxygen, Followed by the Inhalation of O_2 from a Cylinder (Cf. Fig. 3, Parts A, B, and C)

Mean curve: 5 subjects

Time (*min.*)	Log *I*	O_2 in chamber (*per cent*)
Normal air		
2	6.22	20.94
4	5.90	
6	5.75	
7	5.71	
8	5.68	
9	5.55	
10	5.20	
12	4.56	
14	4.10	
16	3.74	
18	3.49	
20	3.33	20.94
22	3.18	
24	3.09	
26	3.03	
28	2.98	
30	2.93	
32	2.89	
34	2.88	
36	2.87	
38	2.86	
40	2.86	20.94
Decreasing concentration of O_2		
2	3.01	12.6
4	3.03	
6	3.05	12.2
8	3.07	
10	3.08	
12	3.09	
14	3.10	11.4
16	3.11	
18	3.12	
20	3.13	
24	3.15	
28	3.18	11.0
36	3.22	

Subject I. L.

Time (*min.*)	Time (*sec.*)	Log *I*	O_2 in chamber (*per cent*)
Normal air			
0	13	7.35	20.94
1	45	6.33	
3	00	6.10	
4	55	6.04	
7	15	5.93	
9	20	5.58	
11	40	4.85	
14	40	4.02	20.94
17	25	3.57	
20	20	3.24	
23	45	3.11	
27	15	2.99	
30	45	2.91	
33	40	2.93	
36	55	2.91	
40	05	2.88	20.94
Decreasing concentration of O_2			
1	10	3.09	13.2
2	35	3.09	
4	00	3.11	
5	10	3.09	
8	20	3.15	
11	05	3.19	
13	05	3.30	
15	20	3.28	11.6
19	05	3.36	
22	40	3.32	
24	45	3.40	
27	00	3.36	
30	10	3.44	11.6
34	10	3.28	
36	20	3.30	
38	30	3.38	
40	40	3.36	
42	50	3.44	
45	10	3.36	11.2

Subject W. F.

Time (*min.*)	Time (*sec.*)	Log *I*	O_2 in chamber (*per cent*)
Normal air			
0	09	7.35	20.94
1	50	6.31	
3	30	5.91	
5	45	5.87	
7	50	5.66	
10	15	5.10	
12	20	4.53	
14	00	3.88	20.94
16	30	3.63	
19	40	3.19	
22	40	3.13	
25	30	3.05	
29	05	2.88	
32	50	2.82	
37	05	2.78	
40	20	2.78	20.94
Decreasing concentration of O_2			
5	05	3.03	12.3
8	05	2.88	
11	30	3.01	
15	35	2.86	10.9
19	40	3.01	
24	20	3.07	
30	30	3.13	9.6
36	15	3.22	
39	40	3.19	
42	25	3.17	
46	20	3.22	8.5
50	05	3.28	
52	40	3.19	
54	45	3.28	
58	20	3.26	
60	10	3.34	
62	15	3.40	
65	05	3.36	
68	10	3.48	

TABLE III—*Concluded*

Mean curve: 5 subjects		
Time	Log *I*	O_2 in chamber
min.		*per cent*
Decreasing concentration of O_2		
38	3.23	
42	3.24	10.2
44	3.29	
46	3.30	
48	3.31	
50	3.33	
52	3.35	
54	3.36	
56	3.39	
58	3.40	
60	3.42	
After inhaling O_2 from a cylinder		
2	2.92	
4	2.80	
6	2.73	
8	2.73	

Subject I. L.			
Time		Log *I*	O_2 in chamber
min.	*sec.*		*per cent*
Decreasing concentration of O_2			
48	05	3.40	
51	00	3.48	
54	20	3.51	
55	40	3.46	
58	10	3.38	
61	05	3.48	10.8
After inhaling O_2 from a cylinder			
1	05	3.09	
1	35	2.95	
2	10	2.99	
3	05	2.88	
4	00	2.91	
5	10	2.78	
6	05	2.74	

Subject W. F.			
Time		Log *I*	O_2 in chamber
min.	*sec.*		*per cent*
Decreasing concentration of O_2			
70	05	3.51	
74	10	3.55	
78	00	3.57	7.3
After inhaling O_2 from a cylinder			
1	05	2.86	
1	30	2.82	
2	10	2.68	
2	40	2.66	
3	00	2.68	
5	10	2.68	
6	00	2.64	

12,500 feet altitude. During the following hour the threshold was determined every 2 to 3 minutes as the oxygen in the chamber was being reduced by a slow but continuous inflow of nitrogen. The final level of the oxygen averaged 10.2 per cent in five of the experiments (*cf.* Fig. 3A), but in the case of W. F., it was lowered to 7.3 per cent (Fig. 3C).

In each experiment, the threshold gradually rose as the concentration of oxygen decreased. In interpreting the results, one should keep in mind the fact that the data are plotted in logarithmic terms. The mean increase in the threshold, if plotted in micromicrolamberts instead of in their logarithms, was approximately fivefold for the five subjects of Fig. 3A and for subject I. L. in Fig. 3B, and eightfold for subject W. F. in Fig. 3C. The regeneration of the photochemical substances was apparently complete at the end of 40 minutes' dark adaptation. However, with gradually decreasing O_2 concentrations the thresholds continued to rise in proportion to the degree of anoxia. Although anoxia may impair the photochemical processes in the retina in some unknown way, it is more probable that the effects

are on the nervous tissue involved in the visual mechanism either in the brain or periphery or both. This will be discussed more fully below. The

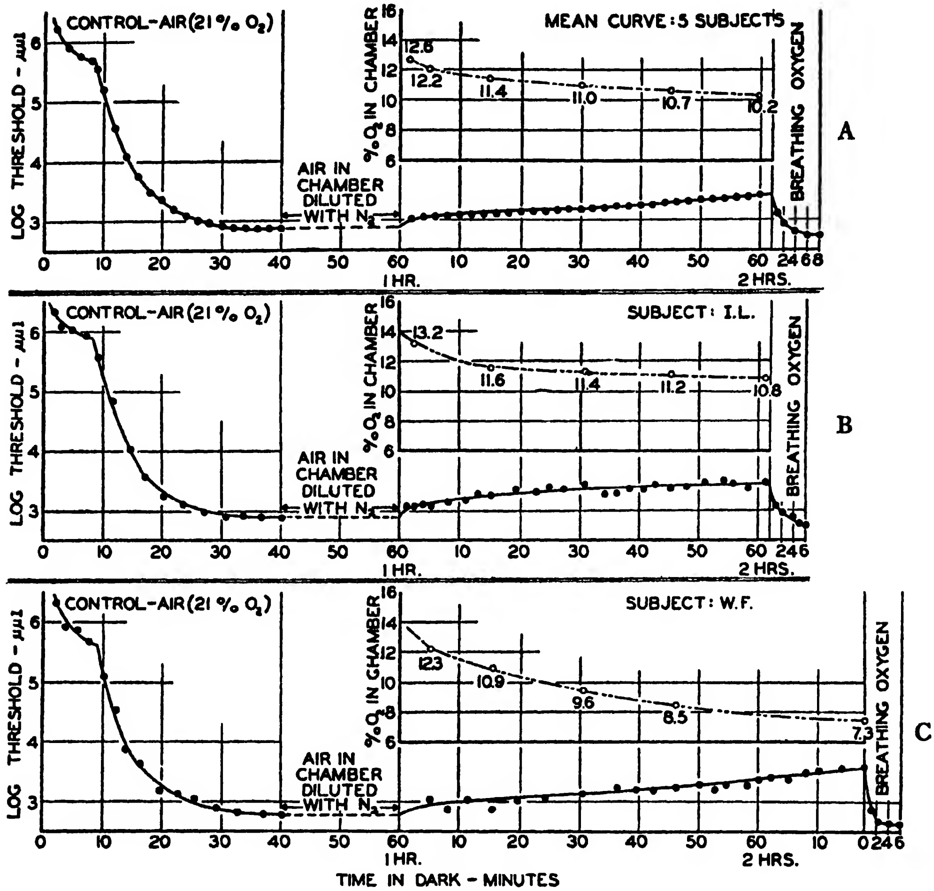

FIG. 3. The effects on light sensitivity produced by altering the percentage of oxygen in the inspired air. After the eye was dark adapted (control curve) for 40 minutes, the air in the chamber was diluted with nitrogen. Then the thresholds, which were determined every 2 to 3 minutes, gradually rose as the concentration of oxygen was reduced. At the end of each experiment, the subject inhaled oxygen from a cylinder and the threshold returned to normal. The curves shown in (A) are based on the mean of 5 subjects. The individual curves of two subjects are shown in (B) and (C). (*Cf.* Table III.)

inhalation of oxygen restored normal function within 2 to 3 minutes, the final threshold usually dropping as low as or lower than the original level.

In Series III the effects of variations in blood sugar on light sensitivity

were studied both in air and in low oxygen. The dark adaptation curves were determined in air and then twice during glucose tolerance tests. The blood sugar determinations are shown in Table IV. The thresholds of light sensitivity for five subjects were not changed significantly during the 1st hour of the tolerance tests while the blood sugar was rising or while it was high, but during the 2nd hour when the blood sugar was falling there was an impairment; *i.e.*, a rise in the threshold above the normal level. The effect of raising the blood sugar by giving a normal breakfast was tested later on a larger group with rather different results.

The procedure was then varied so as to study the effectiveness of hyperglycemia in counteracting the impairment associated with anoxia. Two subjects were tested first in air and then in 10 per cent O_2 and a third time

TABLE IV

The Concentration of Sugar in the Blood during Glucose Tolerance Tests (Mg. Per 100 Cc. of Blood)

	I. L.	W. F.	R. M.	K. N.	S. T.	M. O.	Mean
Control	71	80	89	90	87	84	83.5
25 min.	110	139	152	145	148	127	136.3
1 hr.	95	125	141	125	132	123	123.5
1½ hrs.	80	105	70	124	98	84	93.5
2 hrs.	104	110	84	104	94	92	98.0

while still in 10 per cent O_2 but after ingesting 80 gm. of glucose. During the first two tests the blood sugar was about 70 mg. per cent; during the third over 110 mg. per cent. In each case the light sensitivity was increased after eating the glucose, *i.e.* the threshold fell in spite of the continuing low oxygen but it did not quite reach the normal level.

This line of experimentation was continued but a different procedure was followed. Each of five subjects was given two tests on the adaptometer, the first one being in air, and the second in low oxygen, the average oxygen concentration being 10.4 per cent. While the eyes were completely dark adapted, *i.e.* after the curve had been followed for 40 minutes, each subject drank a glassful of strong glucose solution (1 gm. glucose per kg. body weight in 200 cc. of water). The results for M. V. are shown in Fig. 4 and Table V and for four additional subjects in Fig. 5 and Table V. It took 4 minutes to consume the glucose and from 6 to 8 minutes elapsed as indicated by the arrows in Figs. 4 and 5 before additional thresholds were determined in low O_2. The blood sugar averaged 65 mg. per cent during the low oxygen tests and remained fairly constant as shown in Table VI.

These values rose to an average of 128 mg. following the ingestion of the glucose. It is interesting that of this group the effects of low O_2 on light

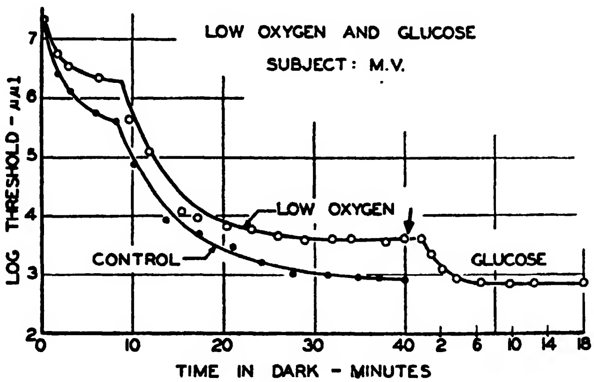

FIG. 4. The effects of low oxygen and glucose on the dark adaptation curve for Subject M. V. The solid circles (control curve) are based on measurements in normal air and the open circles in 10.4 per cent O_2 simulating 18,000 feet altitude. The effects of the anoxia were counteracted by the ingestion of glucose (80 gm.). (*Cf.* Tables V and VI.)

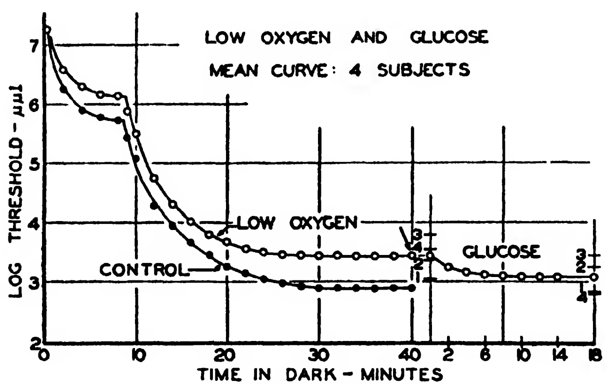

FIG. 5. The effects of anoxia and glucose on the average dark adaptation curve for four subjects. The solid circles (control curve) are based on measurements obtained in normal air and the open circles in concentrations of O_2 averaging 10.4 per cent. The extent to which glucose counteracted the effects of anoxia in each individual is shown in the difference between the numbered lines at the end of the graph. Each number represents an individual subject. (*Cf.* Tables V and VI.)

sensitivity were the least in the case of the subject No. 2, who had showed a significant increase in blood sugar during the control test possibly due to excitement (*cf.* Table VI and subject 2 in Fig. 5). The light sensitivity was improved in each subject after ingesting the glucose in low oxygen, although

TABLE V

Thresholds of Light Sensitivity in Normal Air, Compared with Those in Low Oxygen before and after the Ingestion of Glucose (Cf. Figs. 4 and 5)

Subject M. V.						Mean of four subjects		
Control in normal air (20.94 per cent O_2)			10.4 per cent O_2 (18,000 ft.)			Time	Control in normal air (20.94 per cent O_2)	10.4 per cent O_2 (18,000 ft.)
Time		Log *I*	Time		Log *I*		Log *I*	Log *I*
min.	*sec.*		*min.*	*sec.*		*min.*		
0	14	7.35	0	19	7.35	2	6.26	6.59
1	50	6.41	1	40	6.74	4	5.90	6.30
3	10	6.12	3	00	6.53	6	5.78	6.16
6	00	5.77	6	30	6.33	8	5.74	6.14
8	20	5.60	9	40	5.62	10	5.08	5.50
10	15	4.88	12	00	5.10	12	4.29	4.76
13	50	3.92	15	35	4.02	14	3.96	4.31
17	30	3.69	17	30	3.96	16	3.68	4.01
21	00	3.46	20	25	3.82	18	3.45	3.80
24	10	3.24	23	10	3.77	20	3.26	3.68
27	30	3.01	26	05	3.67	22	3.14	3.56
31	30	2.99	29	00	3.61	24	3.05	3.50
34	50	2.95	32	10	3.63	26	2.98	3.46
37	10	2.95	34	20	3.61	28	2.92	3.45
40	15	2.91	38	05	3.57	30	2.91	3.45
			40	10	3.61	32	2.90	3.44
						34	2.90	3.44
						36	2.90	3.44
						38	2.89	3.44
						40	2.89	3.44
			After ingesting glucose (75 gm.)					After ingesting glucose (70–80 gm.)
			2	50	3.36	2		3.23
			3	55	3.09	4		3.17
			5	15	2.88	6		3.13
			8	20	2.86	8		3.12
			11	40	2.84	10		3.10
			14	15	2.86	12		3.09
			20	10	2.82	14		3.09
						16		3.09
						18		3.09

TABLE VI

The Concentration of Sugar in the Blood of Five Subjects in the Basal State during Experiments Carried out in Low Oxygen and after the Ingestion of Glucose (Cf. Figs. 4 and 5) (Mg. Per 100 Cc. of Blood)

		(1) I. L.	(2) Y. O.	(3) M. O.	(4) S. T.	(5) M. V.	Mean
Control in normal air........		71	77	60	63	76	69.4
10.4 per cent O_2	15 min. later..........	62	66	64	55	65	62.4
	40 " "	66	80	69	54	66	67.0
	1 hr. 15 min. later.....	65	78	72	58	60	66.0
	15 min. after 70–80 gm. of glucose..........	120	132	145	116	131	128.8

TABLE VII

Thresholds of Light Sensitivity for Subject W. F. and for a Group of Nine Subjects in Normal Air (20.94 Per Cent O_2); after Injection of Insulin; after Inhaling O_2; after Being Returned to Normal Air; and after Ingesting Glucose (Cf. Figs. 6 and 7)

Subject W. F.						Mean of 9 subjects		
Control (normal air)			Insulin (8 units) (normal air)			Time	Control (normal air)	Insulin (mean-7 units) (normal air)
Time		Log *I*	Time		Log *I*		Log *I*	Log *I*
min.	*sec.*		*min.*	*sec.*		*min.*		
0	08	7.35	0	08	7.35	2	6.23	6.36
1	40	6.26	1	55	6.28	4	5.93	5.07
3	30	6.02	3	40	6.02	6	5.84	5.94
6	00	5.81	5	55	5.87	8	5.76	5.89
8	55	5.62	8	40	5.77	10	5.32	5.60
11	55	4.33	10	35	5.14	12	4.60	4.90
14	50	3.73	12	20	4.79	14	4.14	4.44
16	40	3.51	14	00	4.08	16	3.80	4.10
19	50	3.22	16	10	3.71	18	3.56	3.86
23	05	2.95	18	35	3.46	20	3.38	3.68
27	05	2.82	20	50	3.42	22	3.23	3.56
30	20	2.78	23	10	3.38	24	3.14	3.46
34	15	2.72	27	05	3.24	26	3.07	3.40
37	05	2.68	30	00	3.11	28	3.01	3.36
40	20	2.68	33	05	3.07	30	2.97	3.32
			36	10	3.03	32	2.94	3.28
			39	05	2.99	34	2.93	3.27
			42	10	3.03	36	2.92	3.27
			44	15	3.05	38	2.92	3.27
			48	00	3.01	40	2.92	3.27
			53	00	2.99			

Subject W. F.: After inhaling O_2 from a cylinder			Mean of 9 subjects: After inhaling O_2 from a cylinder		
0	50	2.86	2		3.02
1	30	2.88	4		2.96
2	40	2.68	6		2.95
4	05	2.57			
5	40	2.62			
6	15	2.64			

Subject W. F.: Normal air			Mean of 9 subjects: Normal air		
1	20	2.88	2		3.04
3	00	2.88	4		3.16
			6		3.17
			8		3.16
			10		3.16

Subject W. F.: After ingesting glucose (80 gm.)			Mean of 9 subjects: After ingesting glucose (70 to 80 gm.)		
2	30	2.64	2		3.09
4	00	2.72	4		3.03
7	20	2.68	6		2.98
10	50	2.70	8		2.95
14	05	2.66	10		2.92
17	10	2.68	12		2.91
20	10	2.68	14		2.91

TABLE VIII

The Concentration of Sugar in the Blood following the Injection of Insulin (Cf. Figs. 6 and 7)
(Mg. Per 100 Cc. Blood)

	G. Y.	R. S.	M. V.		W. H.		I. L.			Group mean
	8 units	7 units	5 units	8 units	8 units	8 units	5 units	7 units	8 units	
Control	65	64	85	88	91	77	78	70	79	77.0
22–27 min. after insulin	50	55	77	68	66.5	58	69	66	62	63.5
40–45 min. after insulin	40	54	69	52	31.3	49	61	69	67	54.7
54–60 min. after insulin	43	45	64	60	49	46	68	66	65	56.0
20–25 min. after 70–80 gm. glucose	85	96	125	131	120	105	111	109	118	111.1

TABLE IX

Thresholds of Light Sensitivity in Normal Air (20.94 Per Cent O_2) and after the Injection of Four Units of Insulin While Inhaling 13.2 Per Cent O_2 Simulating 12,000 Feet Altitude (Cf. Fig. 8)

Subject W. F.

Control in normal air (20.94 per cent O_2)			Combined effects of 13.2 per cent O_2 and four units of insulin		
Time		Log *I*	Time		Log *I*
min.	*sec.*		*min.*	*sec.*	
0	06	7.24	0	08	7.35
1	30	6.26	1	20	6.54
3	10	5.89	2	55	6.24
4	55	5.79	3	50	6.22
7	40	5.68	5	30	6.14
10	05	5.10	7	15	6.02
11	55	4.51	8	55	5.98
14	30	3.90	10	35	5.49
17	15	3.34	12	10	5.06
20	45	3.11	14	50	4.43
24	00	3.03	17	50	4.07
26	20	2.88	20	00	3.82
30	15	2.86	23	20	3.48
			26	30	3.57
			28	15	3.46
			30	05	3.46

the changes were not large in subject 2 (*cf.* Fig. 5). We were surprised to find that the light thresholds were affected by the glucose so soon after ingesting it, the sensitivity responding within 6 to 10 minutes.

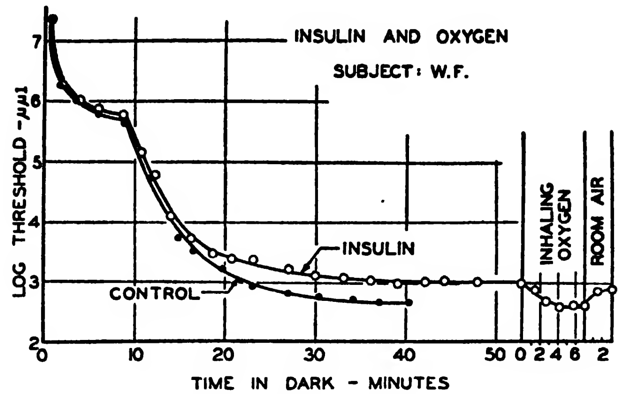

FIG. 6. The effects of insulin (low blood sugar) and oxygen on light sensitivity. The solid circles (control curve) are based on measurements in normal air and the open circles on measurements also taken in normal air but following the injection of insulin. The threshold was increased when the blood sugar was lowered. When O_2 was inhaled at the end of the experiment, the threshold returned to normal but on returning to room air the threshold rose again to the hypoglycemic level. (*Cf.* Tables VII and VIII.)

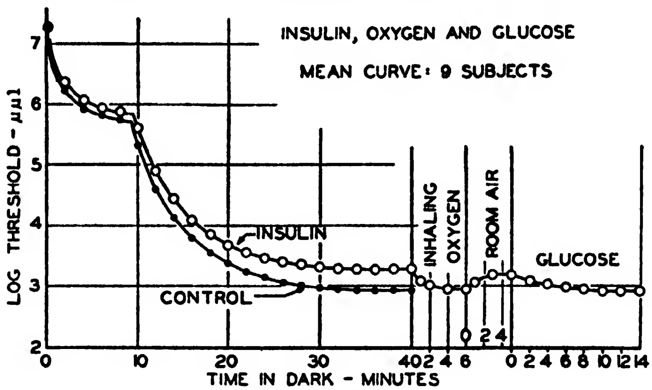

FIG. 7. The effects of insulin, oxygen, and glucose on light sensitivity. The solid circles (control curve) are based on the average thresholds for nine subjects in air and the open circles for the same subjects also in normal air but after the injection of insulin, the inhalation of oxygen, and the ingestion of glucose. When the blood sugar was lowered by insulin the thresholds increased; when the subjects inhaled oxygen from a cylinder, the thresholds returned to normal; when the subjects were returned to room air the thresholds rose; and finally when the subjects ingested glucose the thresholds fell. (*Cf.* Tables VII and VIII.)

In Series IV, the subjects were given insulin followed by the inhalation of oxygen and the ingestion of glucose. The results obtained with the adaptometer are shown in Table VII and Figs. 6 and 7. The procedure was as follows: after the initial observations in the normal state, each sub-

ject was given from 5 to 8 units of insulin intramuscularly (according to body weight). After an interval of approximately 10 minutes, a second dark adaptation period was begun. Samples of finger blood were taken every 10 to 15 minutes for the determination of sugar (Table VIII). The lowest blood sugar values were reached during the testing of the rod portion of the curves so the effects on light sensitivity were naturally greatest during that part of the experiment. At the end of the insulin test, each subject

TABLE X

Data for Subject W. F. Obtained during the Experiment in Which the Combined Effects of Low Oxygen and Low Blood Sugar (Insulin) Were Studied
(Cf. Table IX and Fig. 8)

Conditions	Chamber air		Alveolar air		Blood sugar	Pulse rate	Blood-pressure	Hemoglobin	Code test
	O_2	CO_2	pO_2	pCO_2					
	per cent	*per cent*	*mm. Hg*	*mm. Hg*	*mg. per cent*	*per min.*	*mm. Hg*	*gm. per cent*	*sec.*
Control in normal air	20.96	0.04	106.2	41.2	80	54	114/72	17.4	156
Minutes after injection of 4 units of insulin:									
22					69				
38					64				
43						73	96/80		
62	13.22	0.18	56.4	33.2	70				171
Minutes after ingestion of 70 gm. of glucose:									
30					131				
35						64	106/76		
55	13.34	0.23	48.3	36.6	123			18.6	140

inhaled oxygen for 6 minutes. He was then suddenly changed back to normal room air. Finally, each subject ingested approximately 70 gm. of glucose in 200 cc. of water. An interval of 4 to 5 minutes passed during the ingestion of the glucose at the point in the experiment indicated by the arrow in Fig. 7.

The curves show clearly that the thresholds for light sensitivity depend upon the concentrations of blood sugar and the partial pressure of oxygen. After inhaling oxygen, for example, the thresholds quickly dropped to the normal base line. If the subject was switched back from pure oxygen to room air, however, the thresholds returned to the level reached during the

insulin test. Finally, if the insulin hypoglycemia was counteracted with glucose, the thresholds returned to normal.

In Series V the combined effects of low oxygen and low blood sugar on the dark adaptation curve were analyzed in the case of Subject W. F. After the control observations, the air in the chamber was diluted with nitrogen until the oxygen was reduced to 13.2 per cent simulating an altitude of 12,000 feet. The subject was then given four units of insulin intramuscularly. 15 minutes later the dark adaptation test was repeated, thereby testing the effects of both anoxia and hypoglycemia. These combined effects on light sensitivity raised the threshold 0.6 of a log unit, a change

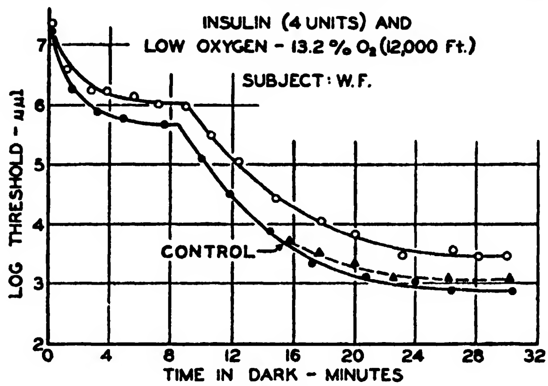

FIG. 8. The combined effects of insulin and low oxygen on light sensitivity. The solid circles (control curve) are based on measurements in normal air and the open circles in 13.2 per cent O_2 and after the injection of 4 units insulin. These effects were largely counteracted (as indicated by the triangles) when glucose was ingested, the oxygen remaining at 13.2 per cent. (*Cf.* Tables IX and X.)

considerably greater than a similar degree of anoxia or of hypoglycemia would have brought about separately, and rather greater than the sum of their separate effects. The results are shown in Fig. 8 and Table IX. This was the same subject who, with twice the amount of insulin (*i.e.* eight units) without anoxia, showed a rise of 0.4 (Fig. 6) and who at 20,000 feet showed a rise of 0.6 (Fig. 1). The alteration in the chamber air in relation to the alveolar air is shown in Table X with several additional physiological tests and a code test involving quickness and accuracy of attention. After 70 gm. of glucose were ingested, the subject remaining in the low O_2, the threshold fell almost to the normal level (*cf.* broken curve, Fig. 8).

In the final series of tests (Series VI), ten subjects were given the dark adaptation test while fasting and after their normal breakfast but without coffee or cigarettes. The results are shown in Table XI and Fig. 9. In the

insulin series, we had observed that changes in light sensitivity were present during moderate degrees of hypoglycemia. Since it is well known that the

TABLE XI

Thresholds of Light Sensitivity Based upon the Means of Ten Subjects in the Basal and Non-Basal State in Normal Air (Cf. Fig. 9)

Time	Basal Log *I*	Non-basal Log *I*
min.		
2	6.33	6.28
4	5.93	5.88
6	5.84	5.84
7	5.75	5.62
8	5.50	5.25
9	5.23	4.85
10	4.91	4.52
12	4.37	3.97
14	3.97	3.55
16	3.68	3.25
18	3.47	3.04
20	3.32	2.94
22	3.30	2.87
24	3.20	2.86
26	3.15	2.84
28	3.15	2.84
30	3.15	2.84

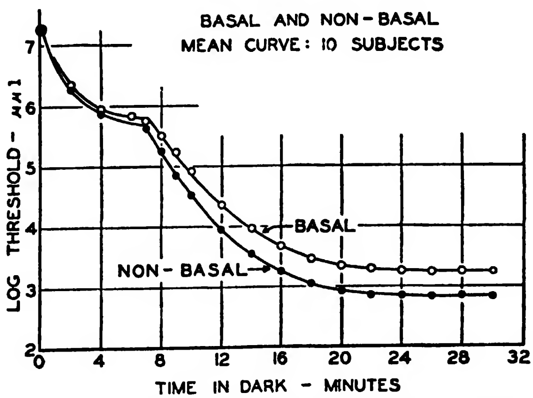

FIG. 9. The mean curves for ten subjects in the basal and non-basal state. (*Cf.* Table XI.)

blood sugar may be as low as 70 to 80 mg. per cent in the morning before breakfast, the curves were plotted under such conditions and repeated after breakfast when the blood sugar had risen to 100 to 120 mg. per cent. In nine out of the ten cases, there was an effect: as shown graphically in Fig. 9 the final threshold in the rod portion of the mean curve was 0.3 of a log unit

higher in the basal state. In our opinion this degree of variation, the amount attributed by Hecht and Mandelbaum (1939) to intra-individual variation during the day or from day to day may be accounted for by the variations in the blood sugar in relation to meals. It is obvious that such a variable should be controlled in psycho-physical experiments involving delicate judgments of visual thresholds.

DISCUSSION

In these experiments we dealt with three primary variables: (1) the functioning of the visual mechanism, judged by the visual threshold and dependent upon oxidative processes in the brain; (2) the tension of oxygen in the inspired (and alveolar) air; and (3) the concentration of glucose in the blood. Our observations suggested that (in crude analogy to the mass action law) the first of these variables is more simply related to the product of the second and third than to either separately. On plotting the results this was found to be the case. Fig. 10 shows the product of the alveolar O_2 tension and the glucose concentration in the blood plotted against the change in the visual threshold observed in the experiment ($\Delta \log I$). The latter is in terms of the increment in the logarithm of the light intensity at the visual threshold, the values obtained with about 100 mm. of alveolar O_2 and 75 mg. of glucose per 100 cc. of blood being used as a base line. This figure includes all the experiments in low O_2 in which the subjects were basal and all but one[2] of those in low O_2 in which glucose was given. The experiments with insulin, with high O_2, and with normal O_2 plus glucose are not included. The correlation between the $\Delta \log I$ and the product of the alveolar O_2 tension and the concentration of glucose in the blood by the Pearson r method was -0.96 ± 0.01. Correlations were also obtained between $\Delta \log I$ and the alveolar O_2 tension and glucose concentration separately; for the former, the correlation was -0.89 ± 0.03 and for the latter -0.87 ± 0.04.

The discrete points in Fig. 10 show a certain amount of scatter, but perhaps less than might be expected in the light of the following considerations. In the first place, the O_2 pressure in the cells of the brain is not necessarily proportional to the tension of O_2 in the alveolar air nor is the glucose at the seat of oxidation necessarily proportional to the blood sugar. Secondly, it was not always possible to keep the alveolar O_2 tension or the concentration of glucose constant while the visual thresholds were being determined during the 30 minute period of dark adaptation. This is particularly true

[2] Experimental difficulties encountered.

of the blood sugar. The results obtained under basal conditions were more consistent than during either the insulin or high glucose series. In fact both the impairment in the thresholds and the variability appeared to increase if the glucose was falling, irrespective of its level. This may be illustrated by the fact that during the part of the glucose tolerance test when the blood sugar was falling rapidly after the large initial increase, a significant rise in the threshold was observed.

Fig. 10, as mentioned above, does not show the points obtained in high O_2, or with normal O_2 and high glucose, in all of which the value for the

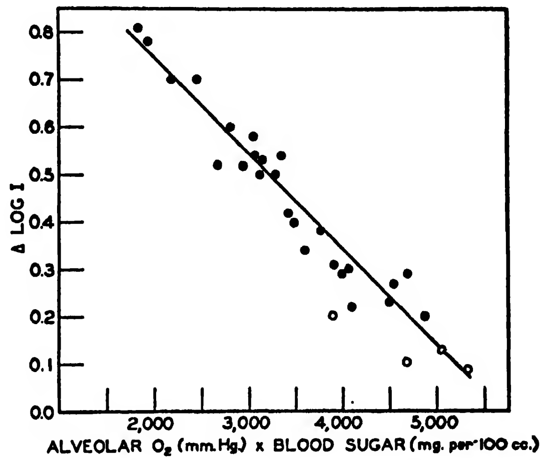

FIG. 10. The difference between the thresholds in normal air (control) and the experimental conditions (low O_2 or low O_2 plus glucose) plotted against the product of the alveolar oxygen tension and the concentration of the blood sugar. The solid circles are results obtained in low O_2 in the basal state. The open circles in low O_2 but after the ingestion of glucose.

abscissa is over 7000. These observations show more scatter but in general fall fairly close to (or just a little below) the horizontal base line even when the abscissa is over 1200; they do not follow the line on the figure which looks as if it would cross the X axis at about 6000. In other words the visual threshold is definitely raised by lowering the product of the O_2 tension and the blood sugar but it is only lowered slightly and inconstantly by raising this product above the normal level. The experiments in which the product was lowered by producing hypoglycemia with insulin while the O_2 tension remained approximately normal gave less constant results than those in Fig. 10 in which the product was lowered by reducing the O_2 tension. The reason for this was probably the considerable changes in the blood sugar which took place during the 30 or 40 minutes of measuring the

thresholds on the adaptometer. Though we took several blood samples, the changing values and the rather less regular curves obtained on the adaptometer in these experiments increased the uncertainty of the data. If put on Fig. 10 all but two of the points would lie above and to the right of the line as drawn. It is as if the blood sugar had actually been about 20 mg. lower than the observed values. The points show far more scatter.

The dark adaptation curves (plotting threshold against time as shown in Figs. 1 and 2) were elevated progressively with diminished O_2 tension; *i.e.*, increased altitude. The curves obtained while breathing low O_2 were similar in shape although consistently elevated throughout the cone and rod portions. The rate of adaptation was apparently unchanged. These experiments were carried out in a chamber where the barometric pressure was constant, the air being diluted with nitrogen to simulate the altitudes as indicated in Figs. 1 and 2 and Table I. When the oxygen was lowered to 13.4 per cent (11,500 feet altitude), the average impairment between the control and low O_2 series at the end of 30 minutes in the dark was 0.26 of a log unit; in 11.5 per cent O_2 (15,400 feet), 0.42 of a log unit, and in 10.1 per cent O_2 (18,500 feet), 0.63 of a log unit. Thus it appears that the impairment in light sensitivity under reduced oxidation is quite large. In Fig. 1, for example, the threshold was raised by a factor 5.8 and in Fig. 2 (in 10.1 per cent O_2) by 6.0. The changes in these experiments are essentially the same as those obtained by McFarland and Evans (1939) under similar experimental conditions, but with a different apparatus. In 13.7 per cent O_2 (11,000 feet) they observed a decrease in threshold of 0.22, and in 11.7 per cent O_2 (15,000 feet) of 0.40 of a log unit. Comparable results have also been reported by Bunge (1936–37) using a rebreathing apparatus, and by McDonald and Adler (1939) with a spirometer. In the former study the rise in threshold was over threefold in 8 to 11 per cent O_2 while in the latter (Hecht adaptometer) both the rod and cone portions of the curve were displaced upward by 0.4 of a log unit (*i.e.* the threshold rose by a factor of 2.5) while inhaling O_2 tensions of 10.4 per cent. Comparable data were obtained by Fischer and Jongbloed (1935–36) and by Clamann (1938) in low pressure chambers indicating that the important variable is the diminished partial pressure of O_2 in the alveolar air whether it is produced by lowering the total pressure or by nitrogen dilution.

In our opinion the effects of anoxia and hypoglycemia on light sensitivity, as shown in Figs. 1 to 7, are exerted on the nervous tissue of the visual mechanism and on the connecting pathways from the retina to the cerebral cortex rather than on the photochemical substances of the receptor cells of the retina for the following reasons. First, in subjects with experi-

mentally induced vitamin A deficiency Hecht and Mandelbaum (1939), Wald *et al.* (1938), and others have shown that there is a greater rise in the rod thresholds compared with the cones. A deficiency of vitamin A is known to affect the regeneration of visual purple relating to night vision and the rods so the latter might be expected to show a greater change. In our experiments, the rod and cone portions of the curves were influenced in essentially the same way, both in extent and in contour suggesting that the effects of vitamin A deficiency and anoxia are dissimilar and that two different processes in the visual mechanism are involved. Additional evidence for this was contributed by McDonald and Adler (1939) for they found that vitamin A deficiency did not alter the effects caused by anoxia. The rise in threshold in anoxia was simply additive and was essentially the same in the normal and vitamin A deficient states. Secondly, the rapidity with which the thresholds change by lowering or raising the alveolar O_2 tension or the blood sugar suggests that the impairment is on the nervous tissue rather than the photochemical processes. Dark adaptation normally takes place within 20 to 30 minutes while in our experiments the final rod threshold could be lowered or raised within 1 to 2 minutes in low O_2 (*cf.* Fig. 1) and low blood sugar (*cf.* Fig. 7) by inhaling oxygen. It is well known that if O_2 is inhaled during experiments involving induced anoxia the arterial oxygen saturation will return to normal within several full inhalations, thereby restoring the O_2 tension in the nervous tissue almost immediately. If the excess O_2 is taken away, the arterial O_2 tension falls very rapidly and in our experiments the threshold returned within 1 to 2 minutes to the former level of impairment. These changes in light sensitivity take place almost as rapidly in hyperglycemia if excess oxygen is inhaled or if the O_2 is restricted (*cf.* Fig. 7). Furthermore, Wald *et al.* (1938) found that at least 7 minutes must elapse before the intramuscular injection of large amounts of carotene affected visual adaptation in vitamin A deficient subjects. Thirdly, we observed that even following complete dark adaptation in normal air for 40 minutes (during which time the regeneration of visual purple should have been complete) the thresholds gradually rose as the oxygen was diminished (*cf.* Fig. 3). The thresholds returned to the normal level, however, within 1 to 2 minutes upon the administration of oxygen. Fourthly, Elsberg and Spotnitz (1938) have reported that the time required for foveal dark adaptation is increased in patients with tumors or other lesions in the cerebral hemisphere. Finally, in experiments reported elsewhere (McFarland, 1932, 1937, 1939), we have observed that anoxia impairs central or cortical functions such as complex reaction times or memory at approximately the same altitudes or under comparable conditions of

oxygen deprivation, suggesting that in both cases the most significant effects are on the central nervous system.

The observations relating to the differences in threshold under basal and non-basal conditions have significant implications in the field of psycho-physics. It is well known that certain psycho-physical laws dealing with delicate sensory judgments, as in light sensitivity, tend to break down at the extremes of the psycho-physical curve. For example, the relationship between sensation and the logarithm of the intensity of the stimulus throughout an extensive intermediate range is linear. At the lowest or highest values of the stimulus, however, significant departures from linearity are known to exist (*cf.* Boring, 1933). Not only does the variability increase so greatly that the smaller differences become statistically insignificant, but also the basis for absolute judgments becomes distorted. The present study illustrates changes in sensitivity at lowest intensities (threshold measurements). Similar effects have also been demonstrated for changes at the highest intensities where visual acuity is maximal (McFarland and Halperin, 1940). If a variation in blood sugar of 20 to 40 mg. per 100 cc. of blood (the usual difference between the basal and the non-basal state) gives rise to a $\Delta \log I$ of 0.31, it would appear that such variables might be controlled to advantage (*cf.* Fig. 9). Since Gellhorn (1936) has shown that raising the tension of CO_2 produces a reversible decrease in visual intensity discrimination poor ventilation of the experimental room might also give rise to equally great changes in delicate sensory judgments of light sensitivity. Hecht and Mandelbaum (1939) suggest that the day-to-day variation in light sensitivity is 0.3 of a log unit. It is possible that the inter-individual variation might be considerably reduced by attempting to control such variables as those mentioned above both in the internal and external environment.

It is improbable that the effects we have reported in these experiments might be due to some artifact, or uncontrolled condition. One such factor to be considered is the size of the pupil. Since it is known that excitement, hyperglycemia, or acute anoxia may give rise to dilation of the pupils, we carried out a number of tests with an artificial pupil. The effects were essentially the same. Bunge (1936–37) whose experiments were carried out under even more acute conditions of anoxia than ours, found by actual measurement of the pupillary changes that they were of such small magnitude that the curves relating to light sensitivity were not significantly influenced. Another possible source of error relates to the insidious effects of acute anoxia and the distortion of judgment or insight into one's own conscious states. In these experiments, the judgments were too con-

sistent and the response of the subjects too prompt for this to be considered of significance. In many instances the subjects were unaware of any subjective symptoms especially in the low blood sugar series and in the less extreme anoxia experiments. Although the variability in the response of the subjects tended to increase under acute anoxia or hyperglycemia, the general characteristics of the curves remained the same.

SUMMARY AND CONCLUSIONS

In this study we have analyzed the effects of variations in the concentrations of oxygen and of blood sugar on light sensitivity; *i.e.* dark adaptation. The experiments were carried out in an air-conditioned light-proof chamber where the concentrations of oxygen could be changed by dilution with nitrogen or by inhaling oxygen from a cylinder. The blood sugar was lowered by the injection of insulin and raised by the ingestion of glucose. The dark adaptation curves were plotted from data secured with an apparatus built according to specifications outlined by Hecht and Shlaer. During each experiment, observations were first made in normal air with the subject under basal conditions followed by one, and in most instances two, periods under the desired experimental conditions involving either anoxia or hyper- or hypoglycemia or variations in both the oxygen tension and blood sugar at the same time.

1. Dark adaptation curves were plotted (threshold against time) in normal air and compared with those obtained while inhaling lowered concentrations of oxygen. A decrease in sensitivity was observed with lowered oxygen tensions. Both the rod and cone portions of the curves were influenced in a similar way. These effects were counteracted by inhaling oxygen, the final rod thresholds returning to about the level of the normal base line in air or even below it within 2 to 3 minutes. The impairment was greatest for those with a poorer tolerance for low O_2. Both the inter- and intra-individual variability in thresholds increased significantly at the highest altitude.

2. In a second series of tests control curves were obtained in normal air. Then while each subject remained dark adapted, the concentrations of oxygen were gradually decreased. The regeneration of visual purple was apparently complete during the 40 minutes of dark adaptation, yet in each case the thresholds continued to rise in direct proportion to the degree of anoxia. The inhalation of oxygen from a cylinder quickly counteracted the effects for the thresholds returned to the original control level within 2 to 3 minutes.

3. In experiments where the blood sugar was raised by the ingestion of

glucose in normal air, no significant changes in the thresholds were observed except when the blood sugar was rapidly falling toward the end of the glucose tolerance tests. However, when glucose was ingested at the end of an experiment in low oxygen, while the subject remained dark adapted, the effects of the anoxia were largely counteracted within 6 to 8 minutes.

4. The influence of low blood sugar on light sensitivity was then studied by injecting insulin. The thresholds were raised as soon as the effects of the insulin produced a fall in the blood sugar. When the subjects inhaled oxygen the thresholds were lowered. Then when the oxygen was withdrawn so that the subject was breathing normal air, the thresholds rose again within 1 to 2 minutes. Finally, if the blood sugar was raised by ingesting glucose, the average threshold fell to the original control level or even below it.

5. The combined effects of low oxygen and low blood sugar on light sensitivity were studied in one subject (W. F.). These effects appeared to be greater than when a similar degree of anoxia or hypoglycemia was brought about separately.

6. In a series of experiments on ten subjects the dark adaptation curves were obtained both in the basal state and after a normal breakfast. In nine of the ten subjects, the food increased the sensitivity of the subjects to light.

7. The experiments reported above lend support to the hypothesis that both anoxia and hypoglycemia produce their effects on light sensitivity in essentially the same way; namely, by slowing the oxidative processes. Consequently the effects of anoxia may be ameliorated by giving glucose and the effects of hypoglycemia by inhaling oxygen. In our opinion, the changes may be attributed directly to the effects on the nervous tissue of the visual mechanism and the brain rather than on the photochemical processes of the retina.

BIBLIOGRAPHY

1. Barcroft, J., The brain and its environment, New Haven, Yale University Press, 1938, chapter III.
2. Boring, E. G., The physical dimensions of consciousness, New York, Century Company, 1933.
3. Bulatao, E., and Carlson, A. J., Influence of experimental changes in blood sugar level on gastric hunger contractions, *Am. J. Physiol.*, 1924, **69**, 107.
4. Bunge, E., Verlauf der Dunkeladaptation bei Sauerstoffmangel, *Arch. Augenheilk.*, 1936–37, **110**, 189.
5. Clamann, G. H., Die Dunkeladaptationskurve des Auges bei Sauerstoffmangel, *Luftfahrtmedizin*, 1938, **2**, 223.

6. Christensen, E. H., Krogh, A., and Lindhard, J., Investigations on heavy muscular work, *Quart. Bull. Health Organization of the League of Nations*, 1934, **3**, 388.
7. Dameshek, W., Myerson, A., and Stephenson, C., The mechanism of the neurological symptoms in insulin hypoglycemia, *Arch. Neurol and Psychiat.*, 1935, **33**, 1.
8. Elsberg, C. A., and Spotnitz, H., The neural components of light and dark adaptation and their significance for the duration of the foveal dark adaptation process, *Bull. Neurol. Inst. New York*, 1938, **7**, 148.
9. Fischer, F. P., and Jongbloed, J., Untersuchungen über die Dunkeladaptation bei herabgesetztem Sauerstoffdruck der Atmungsluft, *Arch. Augenheilk.*, 1935–36, **109**, 452.
10. Gellhorn, E., The effect of O_2-lack, variations in the CO_2-content of the inspired air, and hyperpnea on visual intensity discrimination, *Am. J. Physiol.*, 1936, **115**, 679.
11. Gellhorn, E., Effects of hypoglycemia and anoxia on the central nervous system, *Arch. Neurol. and Psychiat.*, 1938, **40**, 125.
12. Glaze, J. A., Psychological effects of fasting, the effects of prolonged fasting, *Am. J. Psychol.*, 1928, **40**, 236.
13. Hecht, S., Rods, cones and the chemical basis of vision, *Physiol. Rev.*, 1937, **17**, 239.
14. Hecht, S., and Shlaer, S., An adaptometer for measuring human dark adaptation, *J. Opt. Soc. America*, 1938, **28**, 269.
15. Hecht, S., and Mandelbaum, J., The relation between vitamin A and dark adaptation, *J. Am. Med. Assn.*, 1939, **112**, 1910.
16. Himwich, H. E., and Nahum, L. H., The respiratory quotient of the brain, *Am. J. Physiol.*, 1932, **101**, 446.
17. Himwich, H. E., and Fazekas, J. F., The effect of hypoglycemia on the metabolism of the brain, *Endocrinology*, 1937, **21**, 800.
18. Himwich, H. E., Bowman, K. M., Wortis, J., and Fazekas, J. F., Bio-chemical changes occurring in the cerebral blood during the insulin treatment of schizophrenia, *J. Nerv. and Ment. Dis.*, 1939, **89**, 273.
19. Hoagland, H., Rubin, M. A., and Cameron, D. E., The electroencephalograms of schizophrenics during insulin hypoglycemia and recovery, *Am. J. Physiol.*, 1937, **120**, 559.
20. Holmes, E. G., Oxidations in central and peripheral nervous tissue, *Biochem. J.*, London, 1930, **24**, 914.
21. Jowett, M., and Quastel, J. H., Studies in fat metabolism. III. Formation and breakdown of acetoacetic acid in animal tissues, *Biochem. J.*, London, 1935, **29**, 2181.
22. Kerr, S. E., and Ghantus, M., The carbohydrate metabolism of brain. II. The effect of varying the carbohydrate and insulin supply on the glycogen, free sugar, and lactic acid in mammalian brain, *J. Biol. Chem.*, 1936, **116**, 9.
23. Loman, J., and Myerson, A., Studies in the dynamics of the human cranio-vertebral cavity, *Am. J. Psychiat.*, 1936, **92**, 791.
24. McDonald, R., and Adler, F. H., Effect of anoxemia on dark adaptation of the normal and of the vitamin A deficient subject, *Arch. Ophth.*, Chicago, 1939, **22**, 980.
25. McFarland, R. A., The psychological effects of oxygen deprivation (anoxemia) on human behavior, *Arch. Psychol.*, 1932, No. 145, 135 pp.

26. McFarland, R. A., Psycho-physiological studies at high altitude in the Andes. II. Sensory and motor responses during acclimatization, *J. Comp. Psychol.*, 1937, **23**, 227.
27. McFarland, R. A., and Evans, J. N., Alterations in dark adaptation under reduced oxygen tensions, *Am. J. Physiol.*, 1939, **127**, 37.
28. McFarland, R. A., and Halperin, M. H., The relation between foveal visual acuity and illumination under reduced oxygen tension, *J. Gen. Physiol.*, 1940, **23**, 613.
29. Olmsted, J. M. D., and Logan, H. D., The effect of insulin on the central nervous system and its relation to the pituitary body, *Am. J. Physiol.*, 1923, **66**, 437.
30. Olmsted, J. M. D., and Taylor, A. C., The effect of insulin on the blood. I. Changes in oxygen saturation, percentage hemoglobin and oxygen capacity, *Am. J. Physiol.*, 1924, **69**, 142.
31. Quigley, J. P., Johnson, V., and Solomon, E. I., Action of insulin on the motility of the gastro-intestinal tract. I. Action of the stomach of normal fasting man, *Am. J. Physiol.*, 1929, **90**, 89.
32. Wald, G., Jeghers, H., and Arminio, J., An experiment in human dietary night-blindness, *Am. J. Physiol.*, 1938, **123**, 732.
33. Wortis, S. B., Respiratory metabolism of excised brain tissue. II. The effects of some drugs on brain oxidations, *Arch. Neurol. and Psychiat.*, 1935, **33**, 1022.
34. Wortis, J., and Goldfarb, W., A method of studying the availability of various substrates for human brain metabolism during therapeutic insulin shock, *Science*, 1940, **91**, 270.

ENZYMES IN ONTOGENESIS (ORTHOPTERA)

XIII. Activation of Protyrosinase and the Oxidation of Ascorbic Acid*

By THOMAS HUNTER ALLEN and JOSEPH HALL BODINE

(*From the Zoological Laboratory, State University of Iowa, Iowa City*)

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INTRODUCTION

A number of reports showing that quinonoid compounds can act as carriers in the oxidation of ascorbic acid by oxygen have been reviewed by King (1, 2). Since phenol oxidases bring about the production of quinones, the association of ascorbic acid oxidation with these oxidases seems well established. A study of such an oxidation in conjunction with the tyramine-tyrosinase and tyrosine-tyrosinase reactions should be of especial significance in view of the fact that an inactive tyrosinase (protyrosinase) can be obtained from the egg of the grasshopper, *Melanoplus differentialis* (3).

EXPERIMENTAL

Preparation of Protyrosinase.—The procedure for extracting protyrosinase from the grasshopper egg has been described (3). A number of eggs in the diapause stage were ground up and centrifuged in a 0.9 per cent NaCl solution. The fatty layer, which contains an activator of protyrosinase, was removed and the supernatant fluid decanted into a graduated cylinder. To this portion a ½ volume of M/15 KH_2PO_4 solution was added. After standing for several hours at 0°C. this fluid was centrifuged. The clear supernatant liquid, designated B_1 was removed and diluted with a volume of M/15 Na_2HPO_4 solution equal to the amount of KH_2PO_4 solution previously added. The B_1 was next dialyzed at 0°C. against a 0.9 per cent NaCl solution by placing 40.0 ml. of B_1 in a cellophane tube and suspending the tube in 10 to 12 volumes of the saline solution. The latter solution was renewed at the end of each 24 hours. After 3 days the contents of the cellophane tube (volume = 40.7 ml.) were removed and stored at 0°C.

Composition (and Volume) of Reaction Solutions.—The center wells of Warburg manometer vessels contained 0.1 ml. of a 10 per cent KOH solution and a small roll of filter paper. The reaction fluid volume was 3.0 ml. The side bulbs contained 0.5 ml. of the B_1 preparations. 2 ml. of Sorensen's M/15 phosphate buffer solution of a pH = 6.2 were placed in the reaction chamber. In the case of tyrosine this 2.0 ml. portion

* Aided by a grant from The Rockefeller Foundation for work on the physiology of the normal cell.

contained a known amount of tyrosine (Coleman and Bell). The composition of the remaining 0.5 ml. of aqueous solution was varied as to the amount of tyramine hydrochloride (Eastman), the presence of ascorbic acid (Eastman), and an excess of sodium oleate (Merck) or the commercial detergent, Aerosol (American Cyanamid).[1]

RESULTS AND DISCUSSION

The existence of an inactive tyrosinase, which upon the addition of an excess of various activators changes into a tyrosinase, has already been considered (4). It has become simpler to refer to the inactive form as protyrosinase and to the active form as tyrosinase. Throughout this discussion, the behavior of protyrosinase (without activation by sodium oleate or Aerosol) is contrasted to that of tyrosinase (with this activation).

In the presence of undialyzed tyrosinase, tyramine is 0.93 oxidized to melanin in 160 minutes (tyramine, tyrosinase; Fig. 1). A very distinct red color appears within 30 seconds after the commencement of the latter reaction. This color, due to an indole quinone, indicates that the fifth intermediary product of the oxidation of tyramine to melanin is accumulating (5). The uppermost curve (tyramine, ascorbic acid, and tyrosinase) shows an initial rapid uptake of oxygen succeeded by completion of the oxidation of tyramine to melanin. During the first 115 c.mm. oxygen uptake, although the oxidation of tyramine to melanin has started, the time of appearance of the indole quinone red color is delayed for some 8 minutes until the ascorbic acid is oxidized to dehydroascorbic acid. Therefore, the apparent inhibition of tyrosinase, if one views the rate of color formation as a measure of enzyme activity, is probably concerned not with a primary effect upon the enzyme but rather with an alteration in the velocity of formation of intermediary products (6). Judging from both the lack of a red color during these first 8 minutes and from the rapidity of oxygen uptake it seems that the oxidation of ascorbic acid involves the reduction of the quinone of 3:4 dihydroxyphenylethylamine, the third intermediary product in the oxidation of tyramine to melanin (5). In contrast to these two systems the following experiments, also with undialyzed extracts resulted in no observable oxygen uptake: protyrosinase alone; tyrosinase alone; protyrosinase and ascorbic acid; protyrosinase and tyramine; protyrosinase, ascorbic acid, and tyramine (Fig. 1).

The result of dialyzing a B_1 preparation against the sodium chloride solution is graphically illustrated in Figs. 1 and 2. There is no significant change in the velocity of oxidation of 2.3×10^{-3} mM of tyramine in the presence of tyrosinase. Neither does dialysis affect the velocity of oxygen

[1] The authors wish to express their appreciation to the American Cyanamid Company for supplying the Aerosol used in these experiments.

uptake by this amount of tyramine and 0.01 mM of ascorbic acid (equivalent to 112 c.mm. of oxygen) in the presence of tyrosinase. It was also found that no measurable oxygen uptake occurred in the following experiments on dialyzed material; protyrosinase; tyrosinase; protyrosinase and tyramine; protyrosinase and ascorbic acid; protyrosinase, ascorbic acid, and tyramine. However, there is a marked difference in the effect of an undialyzed and a dialyzed tyrosinase upon the oxidation of ascorbic acid (Fig. 1). This particular oxidation of ascorbic acid in the presence of an undialyzed extract is probably due to a coupled reaction with some oxidation product of a naturally occurring substrate. After this substrate has diffused away, the protyrosinase can be activated and the resulting failure to oxidize ascorbic acid indicates the absence of a substance which can act as a carrier between oxygen and ascorbic acid (7). Since the oxygen uptake of dialyzed tyrosinase solutions is not perceptible, it seems that only a minute amount of the natural substrate is needed for the oxidation of ascorbic acid. This is borne out in experiments on dialyzed tyrosinase preparations in which less than 2.3×10^{-8} mM of tyramine still furnishes mM of tyramine still furnishes

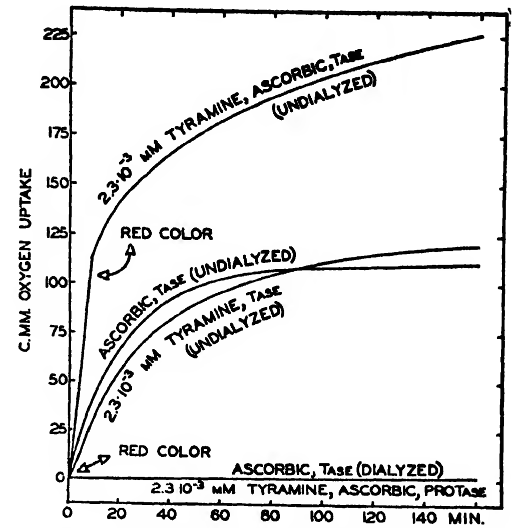

FIG. 1. The effect of dialyzed and undialyzed tyrosinase and protyrosinase preparations upon the oxidation of ascorbic acid. 0.01 mM ascorbic acid; concentration of activator, 0.07 per cent sodium oleate; pH = 6.1; T. = 25.0°C.

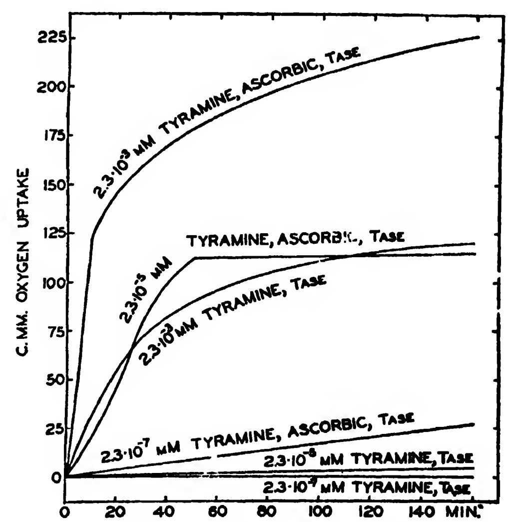

FIG. 2. The effect of dialyzed tyrosinase upon the oxidation of ascorbic acid in the presence of various amounts of tyramine. 0.01 mM ascorbic acid; activator, 0.07 per cent sodium oleate; pH = 6.0; T. = 25.0°C.

enough quinone to catalyze the oxidation of 0.01 mM of ascorbic acid to dehydroascorbic acid (Fig. 2). Obviously the tyramine-tyrosinase reaction with smaller amounts of tyramine oxidizes ascorbic acid at slower rates (Fig. 2).

Lest it be thought that this dialyzable substance be a copper compound which is activated into directly catalyzing the oxidation of ascorbic acid, the following observations should be added. Although the copper-proteinate experiment of Stotz, Harrer, and King (8) could be duplicated, the addition of sodium oleate did not increase the activity of copper with respect to ascorbic acid oxidation in the presence of various amounts of egg albumin. If grasshopper egg tyrosinase is also a copper-proteinate (9–12), one might then conclude *that this oxidase has different* properties from ascorbic acid "oxidase." Such a conclusion seems to be in logical agreement with the distinctions which McCarthy, Green, and King (13) found to exist between ascorbic acid oxidases and catechol oxidase.

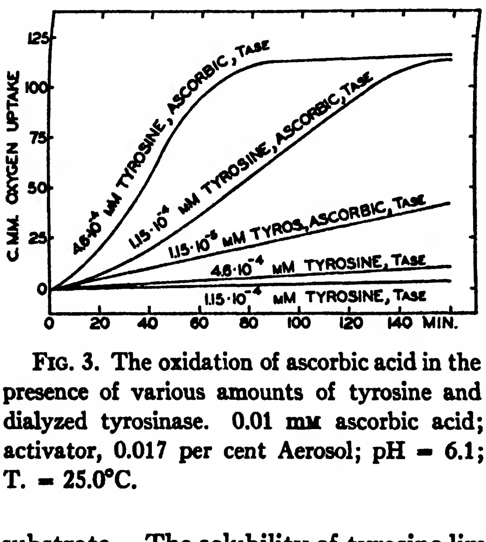

Fig. 3. The oxidation of ascorbic acid in the presence of various amounts of tyrosine and dialyzed tyrosinase. 0.01 mM ascorbic acid; activator, 0.017 per cent Aerosol; pH = 6.1; T. = 25.0°C.

Since the oxidation of small amounts of substrate can be expressed in terms of the readily observed coupled reaction with ascorbic acid, it is possible to use a solution of tyrosine as a substrate. The solubility of tyrosine limits the amount which can be added as a solution, but with concentrations of tyrosine from 4.6×10^{-4} to 1.15×10^{-5} mM (equivalent to 25.8 and 0.7 c.mm. oxygen) there is a coupled oxidation of ascorbic acid (Fig. 3). It was also observed that this reaction with tyrosine and ascorbic acid did not occur unless an excess of Aerosol or sodium oleate was present to function as an activator of the protyrosinase. Neither was there an oxidation of tyrosine in the presence of protyrosinase. Hence, under these conditions, with tyrosine as with tyramine, for a substrate there is still the distinction as to protyrosinase and tyrosinase.

SUMMARY AND CONCLUSIONS

1. Protyrosinase from the egg of the grasshopper, *Melanoplus differentialis*, can be activated by excess sodium oleate or Aerosol.

2. The 3:4 quinone products of the reaction of activated protyrosinase with tyramine or tyrosine will oxidize ascorbic acid to dehydroascorbic acid.

3. The velocity of this latter oxidation of ascorbic acid increases with the amount of tyramine or tyrosine.

4. The oxidation of ascorbic acid by the tyramine-tyrosinase reaction delays the time of appearance of a red color associated with an indole quinone intermediary product in the formation of melanin.

5. Protyrosinase, in itself, and in the presence of tyrosinase substrates does not bring about the oxidation of ascorbic acid.

6. A naturally occurring substrate in a preparation of protyrosinase, sufficient to cause the oxidation of ascorbic acid, can be removed by dialysis against a 0.9 per cent sodium chloride solution.

7. Dialysis against such a solution does not change the properties of protyrosinase; the inactive enzyme must still be activated before it will catalyze the oxidation of tyramine or tyrosine.

8. When the natural substrate, tyrosine, or tyramine is absent, activation of protyrosinase does not result in the oxidation of ascorbic acid.

REFERENCES

1. King, C. G., *Physiol. Rev.*, 1936, **16**, 238.
2. King, C. G., Cold Spring Harbor symposia on quantitative biology, Cold Spring Harbor, Long Island Biological Association, 1939, **7**, 137.
3. Bodine, J. H., Allen, T. H., and Boell, E. J., *Proc. Soc. Exp. Biol. and Med.*, 1937, **11**, 409.
4. Bodine, J. H., and Allen, T. H., *J. Cell. and Comp. Physiol.*, 1938, **12**, 71.
5. Duliere, W. L., and Raper, H. S., *Biochem. J.*, London, 1930, **24**, 239.
6. Evans, W. C., and Raper, H. S., *Biochem. J.*, London, 1937, **31**, 2162.
7. Snow, G. A., and Zilva, S. S., *Biochem. J.*, London, 1938, **32**, 1926.
8. Stotz, E., Harrer, C. J., and King, C. G., *J. Biol. Chem.*, 1937, **119**, 511.
9. Kubowitz, F., *Biochem. Z.*, Berlin, 1937, **293**, 221.
10. Bhagvit, K., and Richter, D., *Biochem. J.*, London, 1938, **32**, 1397.
11. Keilin, D., and Mann, T., *Proc. Roy. Soc. London, Series B*, 1938, **125**, 187.
12. Dalton, H. R., and Nelson, J. M., *J. Am. Chem. Soc.*, 1938, **60**, 3085.
13. McCarthy, J. F., Green, L. F., and King, C. G., *J. Biol. Chem.*, 1939, **128**, 455.

THE EFFECT OF STIMULATION OF THE SENSES OF VISION, HEARING, TASTE, AND SMELL UPON THE SENSIBILITY OF THE ORGANS OF VISION

By FRANK ALLEN and MANUEL SCHWARTZ

(*From the Department of Physics, University of Manitoba, Winnipeg, Canada*)

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HISTORICAL

It has long been known that the stimulation of one sense organ influences in some degree the sensitivity of the organs of another sense. But whether the influence is exerted upon the receptors or upon their central areas in the cortex has not been with certainty determined. This behavior of the nervous system may readily be inferred from its synaptical arrangement and internunciatory constitution whereby all parts are susceptible of communication with each other. These ideas have thus been summarized by Sherrington (12): "All parts of the nervous system are connected together and no part of it is probably ever capable of reaction without affecting and being affected by various other parts, and it is a system certainly never absolutely at rest."

The two senses which seem to be best adapted for the purpose of measurement are those of hearing and vision. As long ago as 1888, Urbantschitsch (13) observed that sounds of different tones may act differently upon the sensitivity of the visual apparatus for various colors, but no definite quantitative relation between sound and color was detected by him. In later investigations Lazarev (11) concluded that the visual sensibility of the retinal periphery, that is of rod vision, increased under the influence of acoustical stimulation of the ear. Yakovlev (15) found that stimulation of the ear by sound conspicuously enlarged the area of the field of cone vision especially for green light. Kravkov (8) observed that under the influence of sound the critical frequency of flicker of white light increases for central or cone vision, and diminishes for peripheral or rod vision.

In a recent investigation Yakovlev (16) has studied in much detail the influence of acoustic stimulation, both by musical tones of frequency 780 cycles per second and noises of 75 decibels in loudness, upon the limits of the areas of the retinal fields for extreme red, orange-red, green, and blue colors. The colors were not spectral but were obtained from Wratten color filters. The maximum transmissions of the filters were at 700 mμ, 680 mμ, 540 mμ, and 440 mμ respectively. Two observers were employed and from their measurements the following results were obtained. Under the influence of both tones and noises the color field for extreme red was unaltered, that for orange-red was diminished, and those for green and blue enlarged in area. Noise was more effective as a stimulus than musical tones, possibly because of its greater intensity, and under its influence the color fields were diminished and enlarged to the greatest extent.

In a more detailed research Kravkov (9) has investigated the influence of acoustic stimulation of the ear upon the light, or rod, and the color, or cone, sensibility of the

visual apparatus. The experiments were performed with the right eye when both eyes were in darkness adaptation. An observer viewed in a spectrometer a small patch of some spectral color which was gradually diminished in intensity by means of an absorbing wedge of neutral tinted glass placed between the slit and the source of light. As the visual field gradually became darker, the observer first indicated the moment when color disappeared, and, second, when light vanished. The light and color thresholds or sensibilities were measured by the reciprocals of the thickness of the part of the wedge in front of the slit at the two positions. The experiments were continued for 1.5 hours, and during this period visual measurements were taken at intervals of from 8 to 13 minutes. After 40 minutes of darkness adaptation a condition of steady visual sensibility was assumed to have been attained, and then an acoustic stimulus, consisting of a musical tone of 2100 cycles per second and 100 decibels in intensity, from a generator of low frequency, was conveyed to both ears of the observer by a telephone receiver for a period of 10 minutes. While the sound was maintained the visual measurements were repeated. At the end of this period the sound was stopped and the measurements were continued as at first. It was found by three observers of normal vision that light (rod) sensibility, contrary to Lazarev's finding, was greatly diminished under the influence of sound. For the colors green and orange-red opposite results were obtained. The sensibility for green (528 mμ) was raised and that for orange-red (610 mμ) was lowered.

The wave-length 560 mμ divides the two effects. For orange-red colors greater than this wave-length the sensibility of the visual apparatus was diminished. For green and blue colors shorter than this intermediate wave-length the sensibility was increased. The ends of the spectrum beyond the wave-lengths 460 mμ and 620 mμ were not observed.

The contradiction between Lazarev's and Kravkov's findings for light, or rod, sensibility may be due to the fact that the observations of the former were made upon the retinal periphery and those of the latter upon the macula. If both sets of observations are correct it follows that the macular and peripheral rods respond in opposite ways to threshold intensities of stimulation.

The Present Investigations

In the present investigations the writers have confirmed the work of Kravkov on the influence of hearing upon color vision, and in addition they have studied the effect of stimulation of the senses of taste and smell upon the perception of colors. They have also extended the observations to include the oscillation of sensitivity of the sensations of vision which results from stimulation of the senses of vision, hearing, taste, and smell.

For convenience of investigation the critical frequency of flicker of the colors of the spectrum was observed before and after the stimulation of the other senses. By comparing the measurements obtained under both conditions, the influence of the other senses upon vision was determined.

The method of experimentation was as follows: A spectrum was obtained from an incandescent lamp of 75 watts which was kept at a steady brightness by a fine rheostat with a voltmeter placed across the terminals of the lamp to insure a constant potential difference. A Hilger spectrometer with the equivalent of three 60° prisms gave a spectrum of wide dispersion, a small portion of which of any desired wave-length was

isolated in the eyepiece by adjustable shutters. Between the lamp and the slit a sectored disk was rotated by an electric motor whose speed was controlled by a leather brake resting upon the axle. To the rear end of the axle was attached a speed-counter which made electric contact every fiftieth rotation of the armature and disk, and the moment of contact was recorded on a strip of paper on a chronograph simultaneously with time indications from a clock beating half-seconds. By measuring these two records, the time of rotation of the disk, and hence the duration of a flash of color upon the retina at its critical frequency of flicker was accurately determined. In making a normal graph of the spectrum for purposes of comparison with those obtained after stimulation of the other sense organs, the eyes were kept adapted to ordinary daylight illumination of the room between the hours of 10 a.m. and 3 p.m. A selected patch of the spectrum whose wave-length was obtained from the calibration curve was viewed in the eyepiece, the disk was rapidly increased in rotation until the critical frequency of flicker was reached, and while this speed was maintained steady by the brake, the record was made upon the chronograph. The sense of hearing, taste or smell, or vision itself, was then adequately stimulated for 2 minutes and the measurements of the critical frequency were repeated immediately or after various intervals of time. This procedure was repeated for colors throughout the spectrum. The graphs for the normal and induced states of vision were then drawn together, as shown in the figures, and from their differences the effect of stimulation of any sense organ upon vision was determined. Since the physical brightness of the spectrum remained unchanged, the differences between the two graphs reveal the physiological changes in brightness, whether increased or diminished, and hence the alterations in responsiveness or sensitivity of the visual sensations, which stimulation of another sense had induced.

The Effect of Stimulation of the Retina upon Color Vision

In making the measurements the normal curve for the critical frequency of flicker was first obtained for the right eye when both eyes were in daylight adaptation. The measurements were then repeated throughout the spectrum with the right eye in constant adaptation for red or yellow light of wave-lengths 687 mμ and 589 mμ. This condition was maintained by stimulating the retina with the red color from a second spectrometer for 2 minutes before each observation of the critical frequency of flicker was taken. Two sets of measurements were made; one, when no rest interval was taken, and, second, when a rest interval of 3 minutes was allowed between the termination of stimulation of the retina and the reading of the critical frequency of flicker. In all other respects the conditions of experiment were alike.

The measurements are given in Table I, and those for the normal and for stimulation by yellow light with no rest interval are shown in graphical form in Fig. 1. The normal is the broken line. The horizontal scale represents wave-lengths and the ordinates are the values of the duration of a flash of light upon the retina at the critical frequency of flicker, or the equal interval of time during which no light is seen. The persistence

of vision carries the impression of one flash of color over the dark interval with no appearance of interruption.

The differences between the two graphs are also shown more clearly in the lower part of Fig. 1. The normal graph is represented by the straight broken horizontal line, and the differences between the two sets of measurements by the continuous line. It is known that the brighter the light the shorter is the duration of stimulation at the critical frequency of flicker. The elevation of the continuous line above the normal, therefore, indicates that the corresponding colors are perceived with diminished brightness, and its depression below the normal shows that the corresponding colors appear enhanced in brightness.

TABLE I

Wave-length	Normal	Stimulation with λ 589 mμ. Rest period = 0		Stimulation with λ 589 mμ. Rest period = 3 min.		Stimulation with λ 687 mμ. Rest period = 0		Stimulation with λ 687 mμ. Rest period = 3 min.	
mμ	sec.	sec.	diff.	sec.	diff.	sec.	diff.	sec.	diff.
720	0.0138	0.0143	+5	0.0134	−4	0.0143	+5	0.0134	−4
700	0.0125	0.0129	4	0.0122	−3	0.0130	5	0.0122	−3
680	0.0116	0.0119	3	0.0113	−3	0.0118	2	0.0114	−2
660	0.0112	0.0112	0	0.0109	−3	0.0112	0	0.0112	0
640	0.0109	0.0109	0	0.0108	−1	0.0107	−2	0.0112	+3
620	0.0107	0.0111	4	0.0105	−2	0.0103	−4	0.0110	3
590	0.0106	0.0112	6	0.0102	−4	0.0101	−5	0.0112	6
550	0.0114	0.0117	3	0.0106	−8	0.0111	−3	0.0117	3
530	0.0119	0.0122	3	0.0113	−6	0.0116	−3	0.0120	1
500	0.0134	0.0132	−2	0.0136	+2	0.0132	−2	0.0137	3
480	0.0156	0.0151	−5	0.0160	4	0.0153	−3	0.0159	3
465	0.0175	0.0169	−6	0.0180	5				

The graphs for stimulation by red light of wave-length 687 mμ, with no rest interval and with a rest period of 3 minutes are shown in Fig. 2 A and 2 B. There is revealed in the latter a complete reversal of the effects of stimulation shown in the former. For the latter graph indicates that the red color is now increased in brightness and the green and violet colors diminished. In other words, the immediate influence of stimulation by red light is to depress the red and enhance the green and violet sensations; while during a rest period of 3 minutes the neural reactions have completely reversed the responsiveness of the sensory apparatus, so that the red sensation becomes enhanced and the green and violet sensations are depressed in sensitivity.

After stimulation by yellow spectral light of wave-length 589 mμ, similar measurements were made with no rest interval and after one of 3

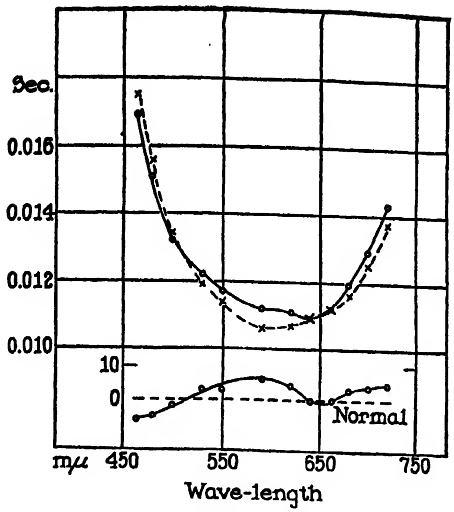

FIG. 1. Effect of stimulation of the retina with yellow light, wave-length 589 mμ, for 2 minutes. No rest interval. The normal graph for the unstimulated retina is the broken line. The lower graph represents differences between the two graphs above.

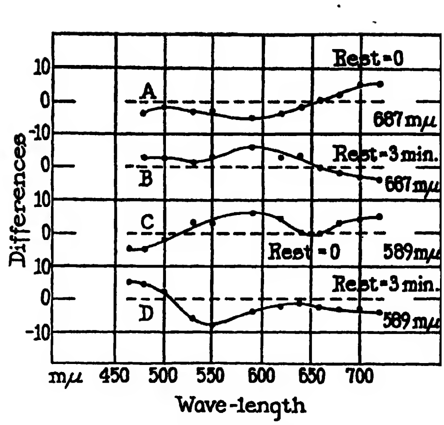

FIG. 2. Effects of no rest interval and of one of 3 minutes after stimulation with red (687 mμ) and yellow light (589 mμ). The effect of rest is to reverse the immediate effect of stimulation. The broken line is the normal.

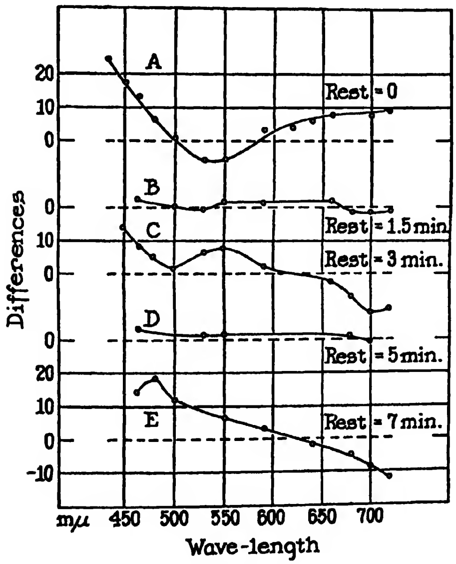

FIG. 3. Visual effect of stimulating the ear with tones of 150 cycles per second, after various rest periods from 0 to 7 minutes. The broken line is the normal.

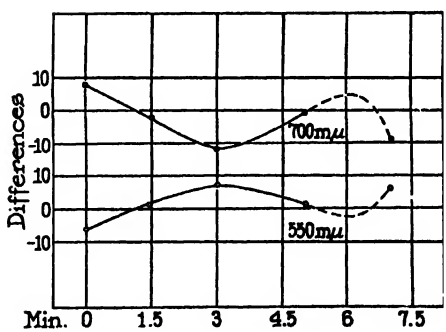

FIG. 4. Oscillation of visual sensitivity after stimulation of the ear by sound. Abscissae are rest periods. Ordinates are differences between normal values of the critical frequency of flicker and the values after various periods of rest. The lines at zero represent normal values.

minutes duration. The data are also given in Table I. The former are shown in Fig. 1 as just described, and they are both shown in the two lower graphs, C and D, in Fig. 2. As the yellow sensation is compounded of the red and green sensations, the graph for no rest interval shows that these sensations have been diminished in sensitivity and the violet has been enhanced by direct stimulation. When rest intervals of 3 minutes were taken after stimulation before readings of the critical frequency of flicker were made, the sensitivities of the sensations were reversed. These reversals occurred through the influence of internal reactions alone.

These observations, as far as the immediate effects obtained with no rest intervals are concerned, confirm the findings of Allen (1) in former investigations.

The Visual Effect of Stimulation of the Sense of Hearing

The influence of stimulation of the sense of hearing upon vision, with which this investigation started, will now be described. For the purpose of stimulation of the ear a Stern Tonvariator was used. This is a Koenig resonator with the bottom like a piston which can be moved inwards to produce a pure tone of any frequency within one octave. The tone, which is generated by blowing a stream of air obliquely across the orifice at the top of the tonvariator, is very pure and free from overtones. In the present investigation two instruments were used, one giving a tone of 150 and the other 1200 cycles per second. Two intensities were used, one given by air pressure of 2 cm. of water and the other by 2 mm. of water. The right ear was held very close to the orifice where the sound was generated, and thus a tone of fairly high intensity was directed into it. The tone given by the lower pressure was very weak. The left ear of the observer was protected from sound by a tuft of cotton wool inserted in the passage, though this precaution, under the conditions of stimulation, was found to be unnecessary.

In making observations the procedure was invariable. The normal curve for the critical frequency of flicker was first obtained by the right eye when the eyes and ears were in normal unstimulated condition, or, more accurately, when both organs were adapted to the daylight and sounds of an ordinarily quiet room. The right ear was then stimulated by the sound for 2 minutes and the readings of the critical frequency of flicker immediately taken. After readjustment of the instruments the aural stimulation was renewed, followed again by the visual measurements. This procedure was repeated until observations were made over the spectrum. The tonvariator was placed near the flicker apparatus so that the

observer could turn immediately from one to the other. Sets of measurements were made with no rest interval, and with rest intervals of 1.5, 3, 5, and 7 minutes between the termination of aural stimulation and the visual observation. The data are given in Table II and are shown graphically in Fig. 3. As before, the broken horizontal lines represent the normal curves, and the continuous lines those for the critical frequency of flicker after aural stimulation. Again, elevations and depressions of the continuous lines indicate respectively diminished and enhanced conditions

TABLE II

Visual Effect of Stimulation of the Sense of Hearing. Stimulation of Right Ear

Wave-length	Normal	Rest period = 0 min.		Rest period = 1.5 min.		Rest period = 3 min.		Rest period = 5 min.		Rest period = 7 min.	
$m\mu$	*sec.*	*sec.*	*diff.*	*sec.*	*diff.*	*sec.*	*diff.*	*sec.*	*diff.*	*sec.*	*diff.*
740	0.0194										
720	0.0170	0.0179	+9	0.0169	−1	0.0159	−11			0.0158	−12
700	0.0150	0.0158	8	0.0148	−2	0.0138	−12	0.0149	−1	0.0141	−9
680	0.0132	—	—	0.0130	−2	0.0125	−7	0.0133	+1	0.0127	−5
660	0.0121	0.0129	+8	0.0123	+2	0.0118	−3	—		—	
640	0.0116	0.0122	+6	—	—	—	—	—		0.0114	−2
620	0.0112	0.0116	+4	—	—	—	—	—		—	
590	0.0106	0.0109	+3	0.0107	+1	0.0108	+2	—		0.0108	+2
550	0.0112	0.0106	−6	0.0113	+1	0.0119	+7	0.0113	+1	0.0118	+6
530	0.0118	0.0112	−6	0.0117	−1	0.0124	+6	0.0119	+1	—	
500	0.0131	0.0132	+1	0.0131	0	0.0132	+1	—		0.0143	+12
480	0.0148	0.0154	+6	—	—	0.0153	+5	—		0.0166	+18
465	0.0165	0.0178	+13	0.0167	+2	0.0173	+8	0.0167	+2	0.0179	+14
450	0.0187	0.0204	+17			0.0201	+14				
435	0.0216	0.0240	+24								

In all cases frequency = 150 vibrations or cycles per sec.
Tonvariator pressure = 2 cm. of water.
Stimulation period = 2 min.

of the brightness of the corresponding colors, which are due to similar changes in the responsiveness of the fundamental color sensations.

The graph in Fig. 3 A, for no rest interval, indicates that the red color of the spectrum appears of lowered intensity, the green of enhanced, and the violet of lowered intensity. This result confirms the experiments of Kravkov, with aural stimulation of 100 decibels in loudness, for red and green colors. He did not carry his measurements into the blue and violet regions. With a rest interval of 1.5 minutes, the graph (Fig. 3 B) shows that the measurements of the critical frequency of flicker are almost of normal value but with a slight indication of a reversal of the condition represented by Fig. 3 A. When a rest interval of 3 minutes was taken,

the graph (Fig. 3 C) reveals that a complete reversal of sensitivity of the red and green sensations has occurred, the red color now appearing brighter and the green dimmer than normal. With aural stimulation, the blue and

TABLE III

Visual Effect of Stimulation of the Sense of Hearing

Wave-length	Normal	Stimulus frequency = 150 cycles per sec. Pressure = 2 cm. Right ear		Stimulus frequency = 150 cycles per sec. Pressure = 2 mm. Right ear		Stimulus frequency = 150 cycles per sec. Pressure = 2 cm. Left ear.	
mμ	*sec.*	*sec.*	*diff.*	*sec.*	*diff.*	*sec.*	*diff.*
740	0.0194			0.0197	+3		
720	0.0170	0.0179	+9	0.0163	−7	0.0166	−4
700	0.0150	0.0158	+8	0.0138	−12	0.0145	−5
680	0.0132	—		0.0127	−5	0.0126	−6
660	0.0121	0.0129	+8	0.0121	0		
640	0.0116	0.0122	+6	0.0118	+2		
620	0.0112	0.0116	+4	0.0115	+3		
590	0.0106	0.0109	+3	0.0111	+5		
550	0.0112	0.0106	−6	0.0115	+3	0.0106	−6
530	0.0118	0.0112	−6	0.0119	+1	0.0113	−5
500	0.0131	0.0132	+1	0.0132	+1		
480	0.0148	0.0154	+6	0.0150	+2		
465	0.0165	0.0178	+13	0.0163	−2		
450	0.0187	0.0204	+17	0.0184	−3		
435	0.0216	0.0240	+24				

Wave-length	Normal	Stimulus frequency = 1200 cycles per sec. Pressure = 2 cm. Right ear		Stimulus frequency = 1200 cycles per sec. Pressure = 2 mm. Right ear	
mμ	*sec.*	*sec.*	*diff.*	*sec.*	*diff.*
740	0.0181	0.0202	+21		
720	0.0166				
700	0.0144	0.0155	+11		
680	0.0134				
660	0.0125	0.0131	+6	0.0128	+3
640	0.0122				
620	0.0114	0.0119	+5	0.0119	+5
590	0.0109	0.0113	+4		
550	0.0118	0.0112	−6	0.0112	−6
530	0.0125	0.0117	−8	0.0116	−9
500	0.0144	0.0135	−9		
480	0.0163	0.0165	+2	0.0159	−4
465	0.0184	0.0187	+3		

violet colors experience no reversal in brightness. After a rest interval of 5 minutes, the brightness of the spectrum, as shown by Fig. 3 D, appears of normal value. When the rest interval was increased to 7 minutes (Fig. 3 E) the curve indicates much the same changes in color intensities as shown in Fig. 3 C for 3 minutes of rest.

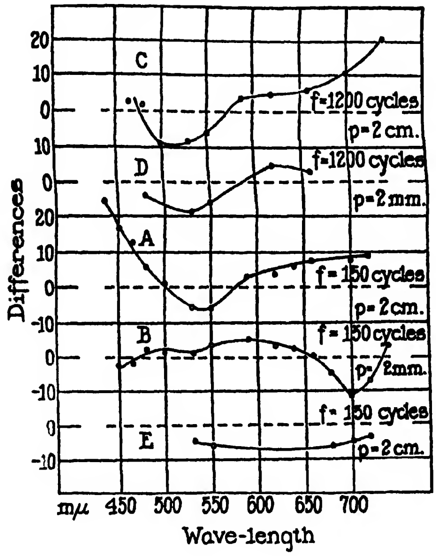

FIG. 5. Visual effects of aural stimulation by tones of different frequencies and intensities. Graph E is contralateral effect of aural stimulation. The broken lines are the normals.

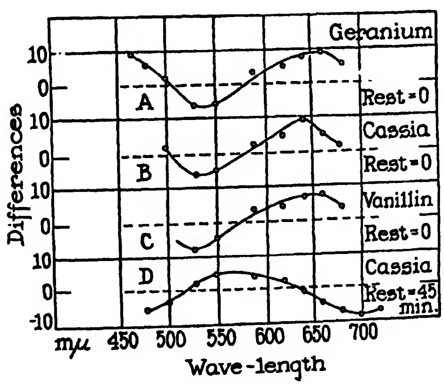

FIG. 6. Visual effect of olfactory stimulation with various substances, after no rest periods and after 45 minutes of rest. The last shows reversal of effect. The broken lines are the normals.

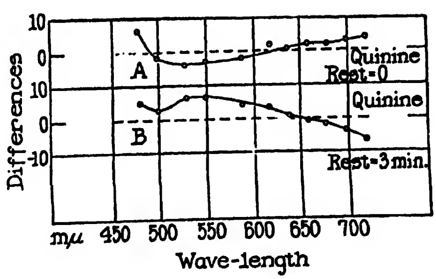

FIG. 7. Visual effect of gustatory stimulation with solution of quinine sulfate, after no rest period and after 3 minutes. Reversal of effect is shown. Broken lines are normals.

Thus by internal reactions alone which are inherent somewhere in the visual apparatus, reversals of sensitivity of the visual organs occur in a definite oscillatory manner. By plotting cross-sections of the five curves

in Fig. 3, the oscillatory effect is more strikingly displayed. This has been done in Fig. 4 for the wave-lengths 700 mμ, and 550 mμ. It will be noticed that the oscillations of sensitivity of the red and green sensations are opposite in phase.

In order to study the influence of aural stimulation of different intensities, a graph was obtained after stimulation by a weak tone of 150 cycles per second, produced by a low pressure of only 2 mm. of water. The data are given in Table III, and are plotted in Fig. 5 B in contrast with Fig. 5 A which is a repetition of Fig. 3 A for 150 cycles per second and a pressure of 2 cm. It will be seen that aural stimulation by the weaker tone has evoked a reversal of the visual effect caused by the louder tone of the same frequency, including, in this case, the violet end of the spectrum.

With aural stimulation by a high-pitched tone of 1200 cycles per second of strong and weak intensities given by air pressures of 2 cm. and 2 mm. of water respectively, the visual effects were those shown in Fig. 5 C and 5 D. These graphs are plotted from the measurements in Table III. They both show depression of sensitivity of the red and probably of the violet sensations, but enhancement of the green. There is no evidence of reversal of visual sensitivity under the influence of the weaker tone. It is possible that for the higher and more piercing tones, a still lower intensity than that obtained with a pressure of 2 mm. of water is required to evoke reversals of visual sensitivities, or else with stimulation by very high tones only depression of sensitivity occurs.

A few readings were obtained to show the contralateral influence of aural stimulation of the left ear upon the right eye. The measurements are given in Table III, and shown graphically in Fig. 5 E. All colors in the range observed from 530 mμ to 720 mμ are seen to be enhanced in brightness.

The Visual Effect of Stimulation of the Sense of Smell

In order to study the influence of stimulation of the sense of smell upon color vision, a volatile odorous material was placed in a bottle through the rubber stopper of which two glass tubes were passed. One of them dipped below the surface of the liquid. A current of air was then gently blown through it which conveyed a steady stream of odorous material through the second tube to the right nostril. The sense of smell was then stimulated by the odor for 2 minutes and readings of the critical frequency of flicker taken immediately afterwards with no rest interval. Three substances were used, oil of African geranium, oil of cassia, and an alcoholic solution of vanillin, all of which gave the same result. The measurements

are given in Table IV and are plotted in Fig. 6, A, B, and C, respectively. With the odor of oil of geranium as the stimulating substance, the red and violet sensations, as shown in Fig. 6 A, were depressed in sensitivity and the green enhanced. With the other two substances the measurements gave similar results except that they were not extended into the blue-violet region of the spectrum.

TABLE IV

Visual Effect of Stimulation of the Sense of Smell

Wave-length	Normal	Stimulation with oil of geranium. Rest interval = 0		Stimulation with oil of cassia. Rest interval = 0		Stimulation with vanillin. Rest interval = 0	
mμ	*sec.*	*sec.*	*diff.*	*sec.*	*diff.*	*sec.*	*diff.*
680	0.0134	0.0140	+6	0.0136	+2	0.0138	+4
660	0.0125	0.0134	+9	0.0130	+5	0.0132	+7
640	0.0119	0.0127	+8	0.0128	+9	0.0126	+7
620	0.0114	0.0119	+5	0.0119	+5	0.0118	+4
590	0.0111	0.0114	+3	0.0114	+3	0.0114	+3
550	0.0117	0.0112	−5	0.0112	−5	0.0112	−5
530	0.0125	0.0119	−6	0.0119	−6	0.0117	−8
500	0.0148	0.0150	+2	0.0150	+2		
480	0.0169	0.0175	+6				
465	0.0185	0.0194	+9				

Wave-length	Normal	Stimulation with oil of cassia. Rest interval = 45 min.		Wave-length	Normal	Stimulation with oil of cassia. Rest interval = 45 min.	
mμ	*sec.*	*sec.*	*diff.*	*mμ*	*sec.*	*sec.*	*diff.*
720	0.0138	0.0131	−7	590	0.0091	0.0095	+4
700	0.0125	0.0117	−8	550	0.0097	0.0102	+5
680	0.0113	0.0106	−7	530	0.0102	0.0104	+2
660	0.0106	0.0102	−4	500	0.0117	0.0114	−3
640	0.0100	0.0099	−1	480	0.0130	0.0125	−5
620	0.0094	0.0097	+3				

A number of attempts were made, but without success, to discover whether, with various short rest periods up to 15 minutes, any reversal of color sensitivities occurred as a result of ipsilateral olfactory stimulation. Since in many ways the sense of smell is rather sluggish, it was decided to allow a rest interval of from 40 to 50 minutes after stimulation with the odor of oil of cassia before measurements of the critical frequency of flicker were made. The result showed (Fig. 6 D) that in the prolonged rest interval a decided reversal of sensitivity of all three color sensations occurred. As the graph indicates, the red and violet sensations are enhanced and the green depressed in sensitivity.

The Visual Effect of Stimulation of the Sense of Taste

Two sets of measurements after stimulation of the sense of taste were made, in both of which the stimulating substance was an aqueous solution of sulfate of quinine. This substance was chosen so that the bitter sensation, which is much the most sensitive of the four taste sensations, would be stimulated. A piece of absorbent cotton was soaked in this solution and placed on the back of the tongue for 2 minutes, then it was removed and the reading of the critical frequency of flicker taken. The mouth was then rinsed with water and the stimulation repeated with a fresh piece of cotton. In the first case no rest interval, and, in the second, a rest interval of 3 minutes was allowed between the cessation of stimulation and the measurement of the critical frequency of flicker. The readings are given in Table V and are shown graphically in Fig. 7 A and 7 B. With no rest interval the red sensation is depressed and the green enhanced in sensitivity, while after a rest period of 3 minutes a reversal occurred in which the red sensation was enhanced and the green depressed in sensitivity. In both cases the violet sensation appears to suffer depression of sensitivity.

TABLE V

Visual Effect of Stimulation of the Sense of Taste

Wave-length	Normal	Stimulation with quinine sulfate. Rest interval = 0		Stimulation with quinine sulfate. Rest interval = 3 min.	
mμ	*sec.*	*sec.*	*diff.*	*sec.*	*diff.*
720	0.0144	0.0148	+4	0.0137	−7
700	0.0128	0.0131	+3	0.0124	−4
680	0.0115	0.0117	+2	0.0113	−2
660	0.0109	0.0111	+2	0.0108	−1
640	0.0104	0.0105	+1	0.0104	0
620	0.0100	0.0102	+2	0.0103	+3
590	0.0099	0.0097	−2	0.0103	+4
550	0.0105	0.0102	−3	0.0111	+6
530	0.0110	0.0106	−4	0.0116	+6
500	0.0122	0.0120	−2	0.0124	+2
480	0.0134	0.0140	+6	0.0139	+5

It may be remarked that stimulation with a solution of sugar was tried but with no apparent visual effect. The sweet sensation is, however, the most insensitive of the four gustatory sensations. Possibly a solution of saccharine might have been successful as a stimulant to produce a change of visual sensitivity.

DISCUSSION OF RESULTS

There is in the human body a wide-spread system of nervous channels through which certain effects of stimulation of one organ are conveyed to other organs both similar and dissimilar in character. One of the writers (6) has shown that stimulation of the right foot by a forward pressure against a wall diminishes the magnitude of the post-contraction muscular reflex in the right arm. This is a case of partial ipsilateral inhibition. The opposite effect had previously been investigated by Whisler (14) in a specially complete manner. In his researches he employed the post-contraction of the left leg as the normal response. Immediately after stimulation of the left arm, he found that the responses of the left leg were augmented. He then stimulated both arms simultaneously and found a greater augmentation of the subsequent response of the leg. The preliminary stimulation was then extended to include at once both arms and the right leg, with still greater augmentation of the response of the left leg. Finally, with these three he included stimulation of the muscles of the neck, and obtained the greatest degree of augmentation of the response of the left leg. Similarly, stimulation of different organs by faradic currents, pictures, music, and colors was followed by augmented post-contraction responses of the left leg.

The influence of various types of stimulation upon glandular secretion has been studied by several investigators. Thermal stimulation of the mouth above 55°C. and below 15°C., was found to be effective in exciting the salivary glands to increased activity. Lashley (10) observed that violent chewing of a tasteless substance such as rubber, elicited a very large increase in the amount of saliva secreted. Activity of the salivary glands is also greatly promoted by acids, alkalis, and salts held in the mouth, and also by many kinds of food especially when they are present in the stomach. Mental work also enhances the activity of glandular organs of several types. Inhibitory influences upon the salivary glands arise from violent effort, rapid movement, and prolonged strain. Most people are aware of the dryness of the mouth which occurs in running, in games such as football and tennis, and in athletic sports generally; and it is a common practice to counteract the inhibition of the salivary glands thus produced by the enhancing action of the chewing reflex promoted by the use of gum. Lashley found no salivary influence exerted by visual, auditory, or tactile stimulation under the conditions of his experiments.

Muscular fatigue may depress the memory, while excitement and apprehension are often found to enhance it. Impassioned emotional states

have widespread physical effects. Fear inhibits the flow of saliva as many an inexperienced speaker has found to his discomfiture. Hunger, which is due to the muscular contraction of the stomach, induces weakness of the knees. Many additional instances of the influence of stimulation of one part of the organism upon the responses of other parts can no doubt be found.

In nerves themselves Erlanger and Gasser (7) have found evidence of an oscillation in excitability. After a nerve fibre has been excited by electrical stimulation, the threshold of response falls to a steady state through a series of three oscillations of diminishing amplitudes in which the threshold values are alternately lowered and raised. The period of these oscillations, 0.005 second, is, however, of an order of magnitude much different from those of 3 minutes which are described in this communication. The two values are perhaps scarcely comparable, since the short period oscillations are those of a single nerve fibre, while the long period oscillations are concerned with large numbers of fibres, their receptors, and their cortical terminations.

1. In the present investigation the writers have brought forward evidence of a precise character to show how stimulation of three sense organs influences the responsiveness of vision. It is found that stimulation with red light, sound, quinine, and odors produces by its immediate action much the same effect upon vision, which is the depression of the red sensation and the enhancement of the green; the violet sensation for some reason being sometimes depressed and at other times enhanced in sensitivity. The magnitude of the visual effect seems in all cases to be about the same. Since stimulation of various senses demonstrably affects vision, stimulation of the eyes probably reciprocally affects those senses. Perhaps all sense organs are so interrelated that stimulation of any one of them influences all others either by enhancing or depressing their responsiveness. It cannot therefore be maintained that the sense modalities are wholly independent of each other. While the validity of Müller's law of "specific energy" is not impugned, some modifying power upon the quality of response of one organ is nevertheless exerted by stimulation of other sensory receptors.

2. An examination of the graphs presented above shows that stimulation of each of the senses selected for experimentation has affected the responsiveness of the visual organs in the three parts by which the colors, red, green, and violet, are perceived. These results afford, therefore, a striking confirmation of the provisions of Young's tricomponent theory of color vision which postulates the existence of three fundamental color

sensations, red, green, and violet. It is further shown that these primary sensations are not independent of each other and that they are not all affected in the same way. For while the red sensation is depressed in sensitivity, the green is enhanced. It seems to be impossible to stimulate or influence in any manner a single color sensation alone.

3. It is shown also in the case of stimulation of the ear, that the intensity of the stimulus may be a determining factor in producing enhancement or depression of the sensitivity of the visual sensations. For it was found that loud and weak tones of the same low pitch evoked opposite conditions of responsiveness in the organ of vision. Also, it was shown that stimulation of the left ear evoked an enhanced visual response in the right eye. It was formerly demonstrated by Allen (2), Hollenberg (3), and Weinberg (5) that weak and strong stimulation of the senses of vision, touch, and taste similarly produced opposite effects on the sensibility of the organs directly involved.

4. One of the most outstanding characteristics of the graphs under discussion is the reversal of sensitivity of the visual sensations which they reveal as apparently a function of the duration of the interval of rest between the termination of stimulation of any sense organ and the measurement of the critical frequency of flicker. While in the experiments described in this communication the organ of vision is the only one tested for the oscillatory effect, it is doubtless the case that all the sense organs possess the same remarkable character. This oscillation of responsiveness has been shown by Allen and O'Donoghue (4) to occur in the post-contraction of the arm after both ipsilateral and contralateral stimulation. It seems to be the case, therefore, that when stimulation of any part of the organism occurs, the responsiveness not only of that part but also of all other parts neurally connected with it is disturbed, and the normal resting equilibrium is restored by a short series of oscillations of sensitivity in which the organs are alternately depressed and enhanced in responsiveness or excitability. Though the oscillation appears as a function of time, it is probably a cellular or molecular condition of the central areas that fluctuates in activity.

Since the responsiveness of the sense of vision oscillates after stimulation has occurred, the character of observations or measurements made in such circumstances would appear to depend on the time which has elapsed after the termination of stimulation; or, in other words, on the phase of oscillation which is predominant at the moment. By neglecting this factor, many contradictory observations in experimental investigations in color vision have doubtless occurred.

5. In the study of conditioned reflexes it has been found that new reflexes can be gradually substituted for habitual or unconditioned ones on a very exact and extensive scale. Those reflexes have demonstrated the existence of unused neural channels which connect the cortical areas and to some extent new activities have been built upon them. While much knowledge has been acquired concerning the character of these reflexes and the manner of establishing them, little seems to be known of the neural mechanisms upon which they are founded. The present investigation seems to deal with those modes of behavior of the central organs which lie at the basis of conditioned reflexes. The cerebral cortex has been described by Myers as a vast unravelled complex. The present experiments on the reciprocal actions of central areas seem to constitute an additional method by which material progress can be made in the unravelling process.

6. It may be safely inferred that stimulation of any sense organ influences all other sense organs in their excitability. There results, in consequence, an oscillatory condition of sensitivity which changes in state in each case by internal reactions governed by the lapse of time. The field of consciousness, to the extent in which it is based on the fluctuating responses of a delicately interlocked system of the senses, can scarcely remain constant under the ceaseless impact upon it of stimuli arising from the outer world and from the organism itself. These requirements, therefore, afford some physiological basis for the widely accepted Gestalt system of psychology, in which sensory presentations are not to be regarded as the narrowly restricted phenomena of individual organs, but as perceptual patterns where now one and then another sensation predominates above the rest.

7. One of the writers (Allen) has in numerous researches on sensory activities generally regarded the central organs as unchanging in sensitivity, and the receptor organs as mechanisms which fluctuate in excitability when stimulated. One result of the present experiments is the demonstration that a central sensory area oscillates in sensitivity when the receptors of other sense organs are stimulated, and also when the receptors to which it is directly attached are stimulated. To ascribe those phenomena of vision, in which fluctuation or alteration of intensity of response is concerned, to the retinal receptors exclusively is now clearly seen to be erroneous. Many such phenomena must originate in fluctuations of responsiveness of the central organs. The modification of the hues of contiguous fields of color by their mutual action upon each other, known as simultaneous contrast, is one group of phenomena which would now appear to arise in the central centres of vision and not in the visual receptors in the retina. One cannot, however, arbitrarily assign either to the

peripheral or to the central organs the phenomena of oscillation. All parts of the sensory apparatus, peripheral, intermediary, and central, have important functions to perform in the excitation of sensations. The complete sensory apparatus from periphery to centre must be regarded essentially as a unit; and stimulation, response, and radiating influence upon other organs are to be viewed as but the several aspects of its complete and complex function.

REFERENCES

1. Allen, F., *J. Opt. Soc. America*, 1923, **7**, 583.
2. Allen, F., *J. Opt. Soc. America*, 1926, **13**, 383.
3. Allen, F., and Hollenberg, A., *Quart. J. Exp. Physiol.*, 1924, **14**, 351.
4. Allen, F., and O'Donoghue, C. H., *Quart. J. Exp. Physiol.*, 1927, **18**, 199.
5. Allen, F., and Weinberg, M., *Quart. J. Exp. Physiol.*, 1925, **15**, 385.
6. Allen, F., *Quart. J. Exp. Physiol.*, 1937, **26**, 305.
7. Erlanger, J., and Gasser, H. S., Electrical signs of nervous activity, Philadelphia, University of Pennsylvania Press, 1937, 108.
8. Kravkov, S. V., Action des excitations auditives sur la fréquence critique des papillotements lumineux, *Acta Ophthalmol. Scand.*, 1935, **13**, 260.
9. Kravkov, S. V., The influence of sound upon the light and colour sensibility of the eye, *Acta Ophthalmol. Scand.*, 1936, **14**, 348.
10. Lashley, K. S., The human salivary reflex and its use in psychology, *Psychol. Rev.*, 1916, **23**, 446.
11. Lazarev, P. P., The influence of an acoustic stimulation upon the light sensibility of the eye, *Proc. Acad. Sc. U. S. S. R.*, Nr. 18 A, 1927 (Russian).
12. Sherrington, C. S., Integrative action of the nervous system, New Haven, Yale University Press, 1920, 8.
13. Urbantschitsch, V., Über den Einfluss einer Sinneserregung auf die übrigen Sinnesempfindungen, *Arch. ges. Physiol.*, 1888, **42**.
14. Whisler, R. G., Modification of the post-contraction reflex, Abstracts of Doctors' Dissertations, Ohio State University Press, 1931, No. 7.
15. Yakovlev, P. A., Visual sensations and perceptions, Moscow, 1935, 127 (Russian).
16. Yakovlev, P. A., The influence of acoustic stimuli upon the limits of visual fields for different colors, *J. Opt. Soc. America*, 1938, **28**, 286.

THE RÔLE OF ORGANIC SUBSTRATES IN PHOTOSYNTHESIS OF PURPLE BACTERIA

By JACKSON W. FOSTER*

(*From the Hopkins Marine Station of Stanford University, Pacific Grove*)

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Ten years ago van Niel[1] set forth the concept that the photosynthetic processes in various pigmented organisms may be interpreted as special cases of a generalized photosynthetic reaction expressed as follows:

$$2H_2A + CO_2 \xrightarrow[\text{Radiant energy}]{\text{Pigments and}} (CH_2O) + H_2O + 2A.$$

Carbon dioxide is reduced anaerobically to cell substance and water by the hydrogen donor H_2A through the agency of photosynthetic pigments with the absorption of radiant energy. The hydrogen donor appears at the end of the reaction in the oxidized form. Thus, in the case of green plants the hydrogen donor is water:

$$2H_2O + CO_2 \rightarrow (CH_2O) + H_2O + O_2;$$

in the case of the green bacteria it is hydrogen sulfide:

$$2H_2S + CO_2 \rightarrow (CH_2O) + H_2O + 2S;$$

while in the purple sulfur bacteria (Thiorhodaceae) various reduced sulfur compounds, including $Na_2S_2O_3$, Na_2SO_3, S and H_2S,[1] and even molecular hydrogen[2] act as hydrogen donors:

$$2H_2 + CO_2 \rightarrow (CH_2O) + H_2O.$$

In addition to these inorganic compounds the purple sulfur bacteria can utilize organic substrates during photosynthesis.[1, 3, 4]

* National Research Council Fellow.

[1] van Niel, C. B., *Arch. Mikrobiol.*, 1931, **3**, 1; Photosynthesis of bacteria, in Cold Spring Harbor symposia on quantitative biology, Cold Spring Harbor, Long Island Biological Association, 1935, **3**, 138.

[2] Roelofsen, P. A., On photosynthesis of the Thiorhodaceae, Dissertation, Utrecht, 1935.

[3] Muller, F. M., *Arch. Mikrobiol.*, 1933, **4**, 131.

[4] van Niel, C. B., *Arch. Mikrobiol.*, 1936, **7**, 323.

The ability to use organic compounds links the metabolism of the Thiorhodaceae with that of the Athiorhodaceae, the latter requiring organic substrates, and being unable to develop at the expense of; *e.g.*, H_2S and CO_2 in the light.[5] The photosynthetic nature of the metabolism of such organic substrates by Athiorhodaceae has been firmly established by Gaffron,[6] who showed that molecular hydrogen can also be used by certain representatives of this group. It seemed, therefore, likely that the generalized equation

$$2H_2A + CO_2 \rightarrow (CH_2O) + H_2O + 2A$$

would also represent the general nature of the photosynthetic metabolism of the Athiorhodaceae, and that organic compounds would here fulfill the function of H_2A; *i.e.*, hydrogen donor for the photochemical reduction of CO_2.[1]

So far this concept has rested entirely upon analogies between the various types of photosynthetic reactions. A clear cut demonstration of the function of an organic substrate *merely as hydrogen donor* may be expected only if it can be shown that such a substrate undergoes an oxidation without change in its carbon skeleton.

Among the vast variety of oxidation processes carried out by different microorganisms it is particularly the oxidation of alcohols that stands out as providing many examples of such typical dehydrogenations.[7] Thus if it were possible to find representatives of the Athiorhodaceae capable of using simple alcohols as organic substrates one might hope to show this hydrogen donor function beyond a doubt.

By setting up enrichment cultures in media containing simple alcohols as the main substrate, some 50 strains of purple bacteria have now been obtained in pure culture, all of which are characterized by their ability to develop at the expense of simple alcohols.

A cursory study of the biochemical behavior of some of these strains showed, however, that with primary alcohols an accumulation of the corresponding fatty acids does not occur. In view of the fact that these strains decompose fatty acids much more rapidly than the corresponding alcohols, this is not surprising. On the other hand, the decomposition of isopropanol with the production of acetone by one of these strains led us

[5] Molisch, H., Die Purpurbakterien nach neuen Untersuchungen, Jena, 1907.

[6] Gaffron, H., *Biochem. Z.*, Berlin, 1933, **260**, 1; 1935, **275**, 301.

[7] The oxidation of numerous primary alcohols to the corresponding fatty acids, and of secondary alcohols to the corresponding ketones by *Acetobacter* species are, perhaps, the most widely recognized cases.

to a quantitative study of this process. The results obtained warrant the conclusion that the reaction may be represented by the equation

$$2\ CH_3CHOHCH_3 + CO_2 \rightarrow 2\ CH_3COCH_3 + (CH_2O) + H_2O.$$

It thus furnishes the first incontrovertible evidence that an organic substrate can function exclusively as hydrogen donor in a photosynthetic process.

In this connection it may be pointed out that the oxidation of isopropanol to acetone has been found applicable to studies on the mechanism of the methane fermentation which Barker in 1936[8] had formulated as a CO_2-reduction process in the dark. Independently Schnellen[9] and Barker[10] have now shown that the methane fermentation in the presence of isopropanol may be represented by the equation

$$4\ CH_3CHOHCH_3 + CO_2 \rightarrow 4\ CH_3COCH_3 + CH_4 + 2H_2O.$$

Materials and Methods

The organism used for the experiments was obtained by specific enrichment cultures in a basal medium[11] containing 0.1 per cent isopropanol. Glass stoppered bottles, completely filled with the solution, and inoculated with a small amount of mud were exposed to continuous illumination in a light cabinet at about 30°C. After growth had occurred serial transfers were made, and isolation of the pure culture was achieved by three successive series of shake cultures in the above mentioned medium with 2 per cent agar added (see also footnote 1).

A complete description of the organism will appear elsewhere.

The formation of acetone was demonstrated by distilling the liquid portion of a 7 day old pure culture directly into an HCl solution of 2,4-dinitrophenylhydrazine. The precipitate was recrystallized and identified as acetone-2,4-dinitrophenylhydrazone.

[8] Barker, H. A., *Arch. Mikrobiol.*, 1936, **7**, 404.

[9] Cited by Kluyver, A. J., *Suomen Kemistilehti*, 1939, 5–6, 81.

[10] Barker, H. A., *J. Biol. Chem.*, in press.

[11] The basal medium consisted of

H_2O	
$(NH_4)_2SO_4$	0.1 per cent
Na_2HPO_4	0.06 per cent
$MgSO_4 \cdot 7H_2O$	0.05 per cent
$NaHCO_3$	0.2 per cent
Yeast autolysate	0.2 per cent,

and was adjusted to a pH of 8. The yeast autolysate was prepared according to Orla Jensen.

M.P. (uncorr.) authentic acetone-2,4-dinitrophenylhydrazone	124°C.
Bacterial product	123.5°C.
Mixed M.P.	124°C.
N content. Theoretical for $C_9H_{10}O_4N_4$	23.52 per cent
Found	23.52 per cent[12]

The photosynthetic transformation of isopropanol to acetone with the simultaneous assimilation of CO_2 has been followed quantitatively (1) by analyses of cultures in different stages of growth, and (2) with suspensions of "resting cells," using the Warburg manometric technique.

Analytical Procedures.—Total carbon dioxide, including carbonate and bicarbonate, was determined manometrically; the Van Slyke apparatus was used for analysis of the culture media.

Isopropanol and acetone were determined on a micro scale. A 5 ml. sample was slowly distilled, after acidification with H_2SO_4, into 1 ml. of H_2O kept in an ice bath, the condenser outlet extending below the surface of the liquid. A total of about 3 ml. was collected, and 1 ml. portions of the carefully measured distillate subjected to the action of 2 ml. of 0.1 N $K_2Cr_2O_7$ in 5 per cent H_2SO_4 at 100°C. for 5 minutes in well stoppered containers. Residual dichromate was determined iodometrically; deduction from a blank, treated in the same way with H_2O instead of isopropanol-containing distillate, yields the amount of $K_2Cr_2O_7$ reduced by isopropanol, which is itself quantitatively oxidized to acetone. 1 ml. 0.1 N $Na_2S_2O_3$ corresponds to 3.00 mg. isopropanol. Numerous checks with standard isopropanol solutions gave theoretical results.

The acetone content of the culture media was determined by the Messinger-Goodwin iodometric method[13] directly on the centrifuged culture solution since distillation proved to result in a loss of from 5–15 per cent of the acetone. The small amount of yeast extract present in the culture medium did not reduce a measurable quantity of iodine. However, under the conditions of the Messinger-Goodwin titration isopropanol is partly oxidized by iodine; the amount of iodine used is proportional to the concentration of the alcohol. By experimentally determining the corrections to be applied for various isopropanol concentrations within the range encountered here the method gave highly accurate and reproducible results.

The possibility had to be considered that the bacteria during their development might produce iodine-reducing substances other than acetone, particularly since such products have been found in cultures of Thio-

[12] I am indebted to Dr. A. J. Haagen Smit, of the California Institute of Technology, for the N analysis.

[13] Goodwin, L. F., *J. Am. Chem. Soc.*, 1920, **42**, 39.

rhodaceae.[14, 4] Van Niel[4] has shown that these metabolic products are non-volatile. By running acetone analysès on the culture liquid before and after boiling off the acetone it could be shown that the iodine reduction in the present experiments was due wholly to acetone. The excellent agreement between the acetone produced and isopropanol utilized corroborates this.

Organic carbon determinations were made with the method of ter Meulen and Heslinga.[15]

Experiments on Growing Cultures

A large batch of the sterile nutrient medium with approximately 0.1 per cent isopropanol was apportioned aseptically into a series of 70 ml. glass stoppered bottles. Each bottle, with the exception of every fourth one in the series, was inoculated with one drop of a young culture; the uninoculated bottles served as controls. Additional controls consisted of cultures in the basal medium without added isopropanol. The bottles were completely filled and the necks heavily sealed with paraffin in order to minimize losses of CO_2 during incubation.

After various periods of time bottles were removed for analysis, and determinations of total CO_2, isopropanol, and acetone were made on the contents of each bottle. The results obtained with the uninoculated controls proved conclusively that no changes in composition had occurred during prolonged incubation.

The data for the experiment, representing the analysis of 20 cultures at different stages of development, have been summarized in Table I.

Each horizontal row of figures represents the data obtained with a single culture. The development in the various cultures did not proceed at exactly the same rate, probably due to differences in the inoculum and other as yet uncontrollable factors. This accounts for irregularities such as a greater oxidation of isopropanol after 6 than after 7 days. The amount of acetone formed during the first 4 days of incubation was too small to be determined accurately. The data for the changes in composition of the medium from the sixth day on show conclusively that the disappearance of 1 mole of isopropanol results in the formation of 1 mole of acetone, and in the disappearance of practically 0.5 mole of CO_2, as required by the above equation. It will be seen that even after 27 days' incubation only about 70 per cent of the isopropanol had been oxidized.

The exact nature of the products of CO_2-reduction cannot be determined

[14] Gaffron, H., *Biochem. Z.*, Berlin, 1935, **279**, 1.

[15] ter Meulen, H., and Heslinga, J., Nouvelles méthodes d'analyse chimique organique, Paris, Dunod, 2nd edition, 1932.

on the basis of these analyses. The actual increase in the number of bacteria during the course of the experiments shows clearly that all the different cellular constituents have been synthesized. The elementary analysis of purple bacteria[4] has proven that the overall composition of this cell material is more reduced than carbohydrate. In agreement herewith van Niel[1] and Muller[3] have found that in growing cultures of Thiorhodaceae the CO_2 uptake remains some 10–20 per cent below that required by the

TABLE I

Disappearance of CO_2 and Isopropanol, and Formation of Acetone, in Anaerobic Cultures of a Strain of Purple Bacteria in the Light

The medium initially contained 1.03 mg. CO_2 and 0.965 mg. isopropanol per ml.

Age of culture	CO_2 uptake		Isopropanol disappeared		Acetone produced		Molar ratio isopropanol:CO_2	Molar ratio acetone: isopropanol
	mg./ml.	millimols $\times 10^4$/ml.	mg./ml.	millimols $\times 10^4$/ml.	mg./ml.	millimols $\times 10^4$/ml.		
days								
2	0.005	1.14	0.030	5.00	0.097	16.70	4.38	3.34
4	0.021	4.77	0.059	9.84	0.102	17.58	2.06	1.79
6	0.068	15.45	0.205	34.19	0.203	35.00	2.22	1.02
7	0.065	14.78	0.170	28.34	0.169	29.17	1.92	1.03
9	0.106	24.10	0.293	48.85	0.300	51.70	2.02	1.06
10	0.115	26.14	0.310	51.70	0.319	55.00	1.98	1.06
12	0.169	38.40	0.462	77.00	0.455	78.45	2.00	1.02
13	0.149	33.92	0.417	69.50	0.440	75.85	2.05	1.09
15	0.172	39.10	0.497	82.90	0.479	82.50	2.12	1.00
17	0.226	51.40	0.614	102.2	0.619	106.8	1.99	1.04
19	0.213	48.40	0.571	95.20	0.575	99.10	1.97	1.04
21	0.201	45.70	0.541	90.20	0.532	91.65	1.97	1.02
23	0.267	60.70	0.717	119.4	0.711	122.6	1.97	1.03
25	0.235	53.40	0.615	102.5	0.610	105.1	1.92	1.02
27	0.246	56.00	0.663	110.4	0.655	113.0	1.97	1.02

equation in which it is assumed that the CO_2-reduction yields carbohydrate.[16] One might, therefore, expect a similar discrepancy in the ratio $\frac{\text{Isopropanol used}}{CO_2 \text{ reduced}}$ in the above experiments. However, the situation in this case is considerably more complicated because the culture medium contains other carbon compounds in addition to isopropanol and CO_2 in the form of a small amount of yeast autolysate. This addition is required to secure growth of the Athiorhodaceae, presumably as a source of as yet unknown growth factors. The complex nature of this material, which no

[16] This assumption is made for the sake of simplifying the generalized equation, and constitutes nothing but a first, rough approximation.

doubt contains a number of different hydrogen donors as well, has so far made a satisfactory analysis and better interpretation impossible.

A combustion analysis of the cell material grown in media without and with isopropanol was carried out by centrifuging the cells contained in 40 ml. of culture, after previous acidification to eliminate carbonate and bicarbonate. The results are presented in Table II, together with data for the CO_2-uptake by the organisms grown in the presence of isopropanol. An aliquot of yeast autolysate, burned after evaporation to dryness without acid treatment made possible the computation of the amount of carbon attributable to the yeast autolysate in the original medium.

The close agreement between the carbon dioxide recovered from the cell material, and the amount of carbon dioxide reduced by the bacteria in the isopropanol culture could indicate that in the presence of isopropanol the

TABLE II

Carbon Analyses of Cells Produced in the Absence and in the Presence of Isopropanol

	CO_2 found	CO_2 used
	mg.	*mg.*
Yeast autolysate	10.9	
	10.9	
Cell material from culture without isopropanol	5.5	
Cell material from culture with isopropanol 23 days (Table I)	10.3	10.8

organic constituents of the yeast autolysate are not converted into cell material, were it not for the fact that the liquid fraction contained organic matter other than yeast extract, apparent by the slow formation of a colloidal precipitate upon standing, and probably resulting from autolysis of the bacteria. Its tendency to cling to and climb up the walls of the tubes made its quantitative recovery impossible.[17]

Attempts were made to obtain better carbon recoveries by evaporating 40 ml. of the cultures to dryness in the presence of excess HCl, and determining organic carbon in the residue. The results are summarized in Table III.

Although the carbon recovery was considerably higher, the results are not entirely satisfactory yet. Apparently the evaporation to dryness in the presence of HCl resulted in the loss of part of the constituents of the medium other than isopropanol and acetone. This is shown by the discrepancy of the organic carbon initially present as yeast extract (10.9 mg. per 40 ml.

[17] Similar difficulties have been encountered and reported by Muller.[3]

(Table I)) and the recovery (6.0 mg. CO_2) after evaporation of the isopropanol-free culture. By assuming that the loss of yeast autolysate constituents is the same in cultures with and without isopropanol a correction for this factor yields a carbon balance in which the CO_2 recovery is too high (column A, Table III). It is likely, however, that in the presence of isopropanol and CO_2 more of the yeast extract is converted into cell material. The smaller amount left unchanged at the end of the experiment would thus cause a reduction in the loss during evaporation in the presence of HCl. By applying the maximum correction for the yeast autolysate equivalent (10.9 mg. CO_2), the recovery is too low (Column B, Table III).

TABLE III

Carbon Analyses of Cultures after Evaporation to Dryness in the Presence of Excess HCl

	CO_2 found	CO_2 used	Per cent recovery, corrected	
			A	B
	mg.	mg.		
Culture in medium without isopropanol	6.0			
Corresponding cultures with isopropanol:				
25 days (Table I)	16.8	9.5	$\frac{10.8}{9.5} = 113.8$	$\frac{5.9}{9.5} = 62$
27 days (Table I)	19.0	10.0	$\frac{13.0}{10} = 130$	$\frac{8.1}{10} = 81$
Culture without isopropanol	6.4			
Culture with isopropanol (14 days)	15.9	6.0	$\frac{9.5}{6.0} = 158$	$\frac{5.0}{6.0} = 83.4$

Hence it appears that the fate of the yeast autolysate constituents depends upon the presence or absence of additional substrates. A closer evaluation cannot be attempted at present.

The results show, however, that the inorganic carbon dioxide, which disappeared from the medium during the development of the bacteria in the presence of isopropanol, is converted into organic cell constituents.

Manometric Experiments

The photosynthetic decomposition of isopropanol with reduction of CO_2 was also studied with resting cells which excludes multiplication of the bacteria, and hence makes the addition of yeast extract superfluous, thus eliminating this complicating factor.

The slow rate of oxidation of isopropanol by the bacterium necessitated

the use of dense suspensions of physiologically active cells in these manometric experiments. Preliminary studies showed the desirability of using relatively young cultures grown in isopropanol media. For one complete experiment the cells produced during 5 days in 5 liters of medium were collected by centrifugation, washed, and suspended in about 15 ml. 0.05 per cent $NaHCO_3$ solution. The suspension was equilibrated with oxygen-free nitrogen containing 5 per cent CO_2, and 2 ml. portions pipetted into the main compartment of a number of Warburg vessels with two side arms. One side arm always contained 0.1 ml. 5 per cent H_2SO_4, the other the solutions to be added as substrate. The gas phase consisted of N_2 with

TABLE IV

CO_2-Assimilation of Athiorhodaceae in the Presence of Isopropanol and Acetone

2 ml. suspension per vessel; 0.1 ml. 5 per cent H_2SO_4; 30.0°C.; gas phase N_2 + 5 per cent CO_2.

Contents of second side arm	CO_2 change in gas phase in μl				Bicarbonate content of liquid in μl CO_2			
	Initial	2 hrs.	5 hrs.	7 hrs.	Initial	2 hrs.	5 hrs.	7 hrs.
0.2 ml. H_2O	–			+10	286			302
0.2 ml. $\frac{M}{20}$ acetone	–			+9				301
0.2 ml. $\frac{M}{20}$ isopropanol	–	−49	−63	−63	286	294	294	287

Total CO_2 change in 7 hrs.

Control	+10 + 16 μl = +26 μl
Acetone	+ 9 + 15 " = +24 "
Isopropanol	−63 + 1 " = −62 " ; corrected for control −88 μl

5 per cent CO_2; the vessels were shaken at 30.0°C. over a bank of 60 watt incandescent lights.

After temperature equilibrium has been reached the pressure in the manometers remains practically constant in the absence of substrate. The addition of isopropanol from the side arm causes a slow but regular decrease in pressure which, after some hours, comes to a standstill.

In Table IV the results of a typical experiment are reproduced.

Inasmuch as the bicarbonate content of the liquid does not change appreciably during the experiment, the decrease in pressure shows the course of the photosynthetic CO_2 uptake (Fig. 1).

The cessation of CO_2 uptake is due to the disappearance of the isopropanol. This, and the conversion of the latter into acetone, has been shown by chemical analyses in which 73–84 per cent of the acetone could be re-

covered. Considering the relatively large gas phase, the high temperature at which the experiments were run for many hours, and the volatility of the acetone such recovery may be deemed entirely satisfactory; also from the suspensions to which acetone had been added the recovery was of the same order of magnitude.

The actual CO_2-uptake in these experiments amounts to about 90 μl per 0.01 millimol of isopropanol. The generalized equation requires 112 μl if the conversion of CO_2 would yield carbohydrate.

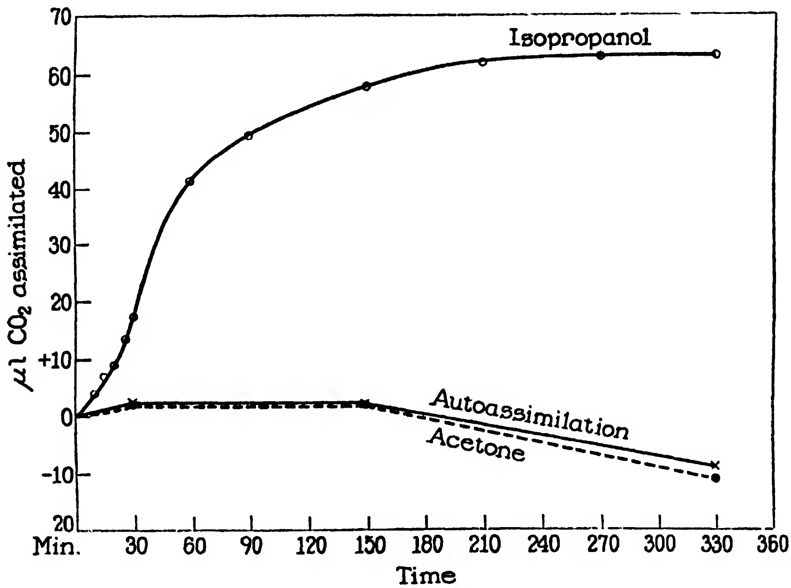

FIG. 1. CO_2 assimilation in the presence of isopropanol and acetone

Experiments on photosynthesis by Athiorhodaceae in the presence of various substrates have shown that the CO_2 uptake is always considerably below this theoretical amount (Gaffron[6]). The average of numerous determinations has shown a discrepancy of 45 μl per 224 μl, computed from the general equation.[18]

Thus the results of the CO_2 assimilation with isopropanol are in excellent agreement with the known facts. The low values for CO_2-uptake must be interpreted to mean that the resting cells do not carry on photosynthesis with the production of carbohydrate, but form more reduced substances.

The results obtained with the suspension in the presence of acetone also

[18] As yet unpublished results of van Niel.

demonstrate conclusively that acetone cannot be used by the organism as a substrate for photosynthesis, and is truly the end product of the isopropanol oxidation. This holds not only for suspensions of resting cells; also culture experiments in the basal medium with acetone proved that growth in such media is never in excess of that which can be obtained in acetone-free medium; *i.e.*, at the expense of the yeast autolysate.

DISCUSSION

The experiments described above illustrate the first case in which the rôle of an *organic* substrate in photosynthesis by Athiorhodaceae can be unequivocally interpreted. Of particular importance is the fact that the transformation of the substrate consists of a simple dehydrogenation, as shown by the stoichiometrical relationship between the substrate and its oxidation product.

The organism itself is of interest also as a representative of a group of Athiorhodaceae capable of using simple alcohols. The purple bacteria previously investigated have appeared inactive with respect to alcohols.[6] The present results show that the contention of Gaffron: "Das Vorhandensein einer Carboxylgruppe ist Grundbedingung dafür, dass ein Körper als Substrat der Assimilation dienen kann . . . ," has become untenable. The discovery of this group of Athiorhodaceae makes it seem probable that among the purple bacteria there would exist a heterogeneity of oxidative biochemical characteristics comparable to that known to occur among the colorless bacteria. But, whereas the latter bring about the oxidation of the substrate with the simultaneous reduction of oxygen, CO_2, nitrate, or sulfate in the dark, the purple bacteria are outstanding in their ability to accomplish such oxidations concomitant with the *photochemical* reduction of CO_2.

The quantitative conversion of isopropanol to acetone with the reduction of an equivalent amount of CO_2 in the light shows the validity of van Niel's generalized equation of photosynthesis

$$CO_2 + 2\ H_2A \rightarrow (CH_2O) + H_2O + 2A$$

beyond a doubt. Also organic substances can thus be used exclusively as hydrogen donors for the photochemical CO_2-reduction.

In a large number of cases the rôle of the organic substrate is not so transparent; usually one observes the complete transformation of organic substances with the simultaneous uptake of CO_2 (or production in the case of highly oxidized substances) into cell materials, and without any detectable remains in the surrounding liquid. This might imply that the cell

constituents are produced exclusively from CO_2, while the organic compound during photosynthesis is completely dehydrogenated (Muller[3]).

However, it would seem premature to draw this conclusion on the basis of the experimental evidence presented above. It is far more likely that in the course of the oxidation of other organic substrates there may be formed intermediate products which can be directly converted into cell materials. (See also van Niel[1] 1935, pp. 140–141.) The recent studies[19] on oxidative assimilation by colorless organisms furnish a very strong support for this view. Wherever the occurrence of such intermediate products is excluded the cell substances must, however, arise from the assimilation of CO_2 alone. The isopropanol oxidation is a case in point.

SUMMARY

A representative of the photosynthetic non-sulfur purple bacteria (Athiorhodaceae) capable of using simple alcohols has been isolated in pure culture.

By means of quantitative analysis of cultures at different stages of development it has been shown that this organism converts isopropanol quantitatively into acetone, simultaneously reducing CO_2 in the light. The data can be represented by the equation

$$2\ CH_3CHOHCH_3 + CO_2 \rightarrow 2\ CH_3COCH_3 + (CH_2O) + H_2O.$$

Manometric experiments with suspensions of resting cells have fully corroborated the results obtained with growing cultures.

The experiments have conclusively proved that an organic substrate may fulfill exclusively the function of hydrogen donor for the photochemical CO_2-reduction in purple bacteria photosynthesis.

I am greatly indebted to Professor van Niel for his constant and invaluable help.

[19] Barker, H. A., *J. Cell. and Comp. Physiol.*, 1936, **8**, 231. Giesberger, G., Beiträge zur Kenntnis der Gattung *Spirillum* Ehbg., Dissertation, Utrecht, 1936; Clifton, C. E., *Enzymologia*, 1937, **4**, 246; Clifton, C. E., and Logan, W. A., *J. Bact.*, 1939, **37**, 523; Winzler, R. J., and Baumberger, J. P., *J. Cell. and Comp. Physiol.*, 1938, **12**, 183; M. Doudoroff, *Enzymologia*, in press.

A NEW FORM OF DIFFERENTIAL MICRORESPIROMETER*

By BURRIS CUNNINGHAM AND PAUL L. KIRK

(*From the Division of Biochemistry, University of California, Berkeley*)

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INTRODUCTION

While more than a dozen microrespirometers have been described in the literature of the past decade, only that devised by Heatley, Berenblum, and Chain (7) is an instrument of general utility like the widely used apparatus of Warburg. While the respirometer of Heatley, Berenblum, and Chain may be used on much smaller amounts of tissue than the standard Warburg apparatus, and is designed to permit mixing of solutions as well as exposure to gases of any desired composition, it is unsatisfactory for the work planned in this laboratory because it does not possess the maximum sensitivity desired by us (0.1 λ/hr.)[1] and is, moreover, rather expensive when the costs of thermostat and optical lever system are included.

In addition to overcoming these objections it was also found that our instrument could be so designed that the chamber size and sensitivity could be simply and rapidly altered to meet the requirements of the diverse materials[2]—protozoan organisms, tissue cells in culture, and tissue slices—to be studied.

It is the purpose of this paper to describe the instrument[3] devised by us as well as to give certain data indicative of the reliability of the measurements obtained with it.

Principle

The respirometer was constructed on the well known differential principle, first used for this purpose by Thunberg (9), and since utilized in modified

* Aided by grants from The Rockefeller Foundation and from the Research Board of the University of California.

[1] 1 λ = 1 $mm.^3$, Kirk, P. L., *Mikrochemie*, 1933, **14**, 1.

[2] The respirometer has also been found useful in studies on insect respiration carried out by Dr. Roderick Craig of the Division of Entomology of the College of Agriculture of the University of California.

[3] Mr. C. E. Camenson of the Microchemical Specialties Co., 2112 Berkeley Way, Berkeley, California, cooperated in the construction of this instrument, and is prepared to supply it.

forms by a number of workers, but especially by Fenn (5, 6) and Barcroft (1, 2).

The essential feature of differential respirometers is the presence of two similar chambers, one of which contains the biological system in its medium, the other an equal volume of medium alone. The two chambers are connected by a piece of capillary tubing carrying an index drop of some liquid of relatively low viscosity and vapor pressure. During the period of measurement the chambers and the connecting tubing constitute a closed system. The consumption of gas in one chamber creates a difference in pressure in the chambers. Equilibrium is restored by the movement of the index drop, which can be measured quantitatively. For many types of work this arrangement is advantageous, permits a cancellation of errors arising from the medium alone, and is independent of external temperature fluctuations, provided that such fluctuations induce the same temperature changes in both chambers. Previously described differential respirometers have failed to take full advantage of this last possibility, which permits *elimination of the thermostat.* Consequently such microrespirometers have required careful temperature regulation.

A disadvantage of the symmetrical differential respirometer is that the displacement of the index drop is only half that which is obtained under similar conditions with a non-differential type. Duryee (4) and Victor (10) have both designed respirometers retaining the closed system feature of the symmetrical differential type, but have made the volume of the chamber containing the biological system negligibly small with respect to that containing none. The displacement of the index drop then approximates the actual change in volume in the respiration chamber. The bulk of the apparatus is thereby considerably increased, since a compensation chamber several hundred times the volume of the respiration chamber is employed. Careful temperature control is necessary since changes in external temperature can hardly be expected to affect both chambers equally, and cancellation of errors arising from the presence of the medium is somewhat less certain, since liquid-gas volume ratios are usually not the same in the two chambers. Consequently, while the apparatus described here may, by a proper choice of fittings, be converted into such a compensated form, it is designed to function primarily as a symmetrical differential type. The symmetrical construction and the placing of the chambers inside a metal block insure a uniform temperature distribution, permit the elimination of the thermostat and make possible a very high degree of sensitivity.

Construction

The respirometer proper and its case were constructed entirely of brass, except for the glass capillaries. The component parts of the respirometer proper and the accessories for its operation can be described under the headings: (1) The chamber block and plugs; (2) the head plate assembly; (3) the respirometer case; (4) accessories.

The construction of the respirometer and its accessories are shown in detail in the accompanying drawing (Fig. 1) in which all dimensions are given in millimeters. A few dimensions could not conveniently be included in the drawing and these will be found in the text below.

1. The Chamber Block and Plugs.—A cylindrical piece of brass 50 mm. in diameter and 35 mm. in length was cut from a brass bar. Two holes, 10 mm. in diameter, were drilled completely through the block parallel to its cylindrical axis. Centers of these holes were 8 mm. from the center of the block face, on opposite sides of the face center. These holes were reamed and polished to the same diameter. The two faces of the block were ground flat against a steel plate, using powdered carborundum and oil. The grinding of one face was continued with fine carborundum and finished with rouge and water against a piece of plate glass.

Three pairs of chamber plugs were constructed of brass cylinders carefully trimmed to fit the holes in the chamber block. The first pair of cylinders were 25 mm. in length, the second 30 mm., and the last 35 mm. In the last pair of plugs (pair C of Fig. 1) circular wells, 4 mm. in diameter and 3 mm. deep, were drilled into the center of one face of each plug. With the last pair of plugs the chamber volume was reduced to 36λ, for handling very small amounts of tissue.

2. The Head Plate Assembly.—A brass disc 6 mm. in thickness was cut from the same brass bar used for the chamber block. Four equispaced holes, 6 mm. in diameter, were drilled to a depth of 4 mm. into the top of this disc. The centers of these holes were located 8 mm. from the center of the disc face. In the center of two opposite 6 mm. holes, 1 mm. holes were drilled through to the opposite lower face of the disc. In each of the other two 6 mm. holes, a pair of 1 mm. holes, 3 mm. apart, were drilled through to the lower face of the disc. The bottom face of the disc was then ground with carborundum and rouge as described under "The chamber block and plugs." The grinding of the contact surfaces of disc and block was completed by grinding the two together with fine rouge, and finally without any abrasive. It was absolutely essential for the proper operation of the respirometer that these two surfaces were ground flat and semi-polished.[4] Failure to achieve this resulted in troublesome "drifting" of the index droplet.

Plugs were inserted into the chamber block and sealed with bakelite varnish at top and bottom. The inside of each chamber was given a very light coat of the same varnish.

Two capillary posts were constructed from brass cylinders 6 mm. in diameter and 15 mm. in length. In each post a 1 mm. hole was drilled through the center of the

[4] Dr. Craig has suggested that the lapping of the surfaces may be more easily achieved if the block and head plate are of metals of different hardness, such as brass and stainless steel.

MICRORESPIROMETER AND ACCESSORIES

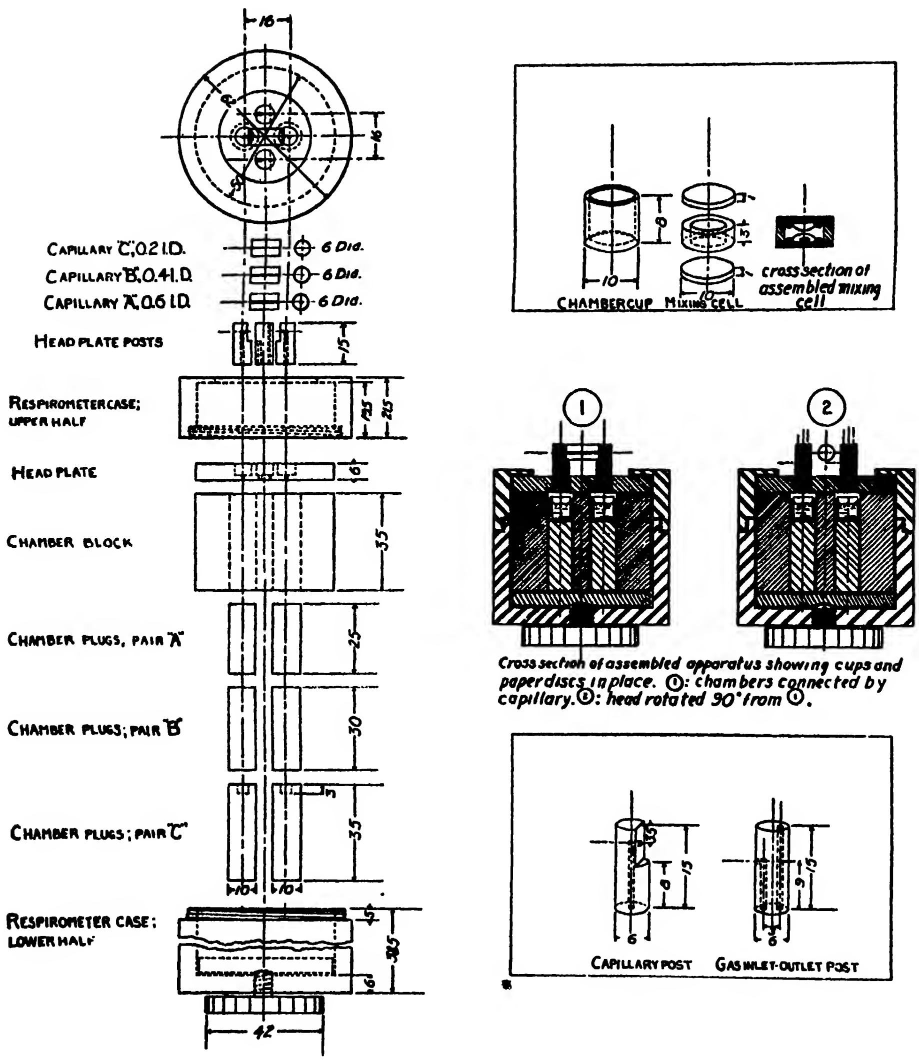

Fig. 1. Scale drawing of microrespirometer and accessories. All dimensions in millimeters. Scale of the two inset drawings is twice that of the remainder of the drawing.

cylinder for a distance of 11.5 mm. after which it was continued at right angles by drilling into the side of the cylinder. The cylinder was filed flat perpendicular to this second hole, to provide a contact surface between capillary and capillary post.

Two gas inlet-outlet posts were constructed similarly, except that one 1 mm. hole

was drilled parallel to the central axis at a distance of 1.5 mm. from it. This extended completely through the post. A second hole was drilled on the opposite side of the center at an equal distance, but only to a depth of 9 mm., after which it was continued at right angles by drilling through the side.

The bottoms of the posts were trimmed to fit the 6 mm. holes in the head plate, as shown in the section drawing of the assembled apparatus. These posts were cemented

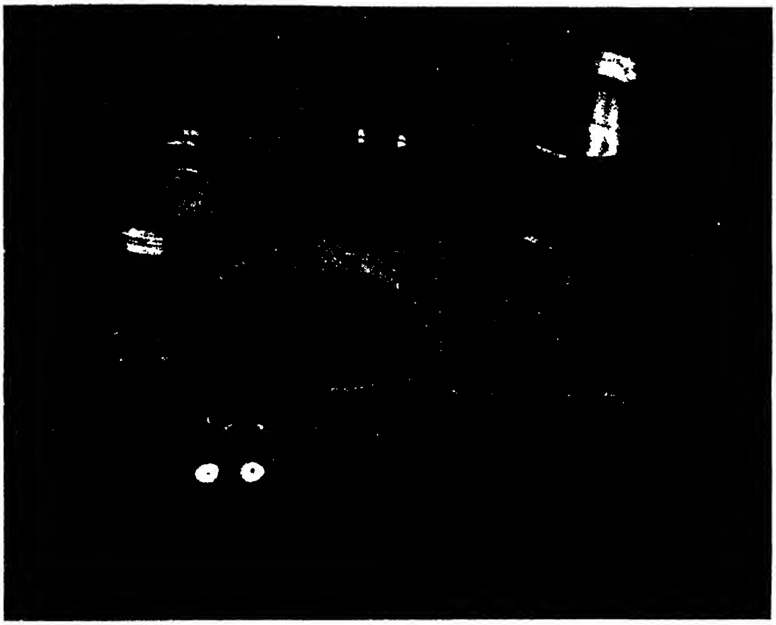

FIG. 2. Microrespirometer and accessories. From left to right: Top row: Respirometer case, upper half; chamber block; respirometer case, lower half.

Second row: Capillaries; head plate assembly; chamber plugs, 25 mm.; chamber plugs, 35 mm.

Third row: Chamber cups; mixing cells with rings and bottom plates assembled.

Bottom row: Absorption discs; mixing bead; top plates of mixing cells.

Note: The respirometer case shown differs in some details from that diagrammed in Fig. 1.

in place with bakelite varnish. The top of each capillary post was given a light coat of shellac and a short piece of small bore rubber tubing was slipped over the end of the post, as may be seen in the photograph (Fig. 2). When the shellac was dry, small holes were drilled through the rubber to connect with the post opening.

Capillaries were constructed from capillary and thermometer tubing cut to the proper length and ground flat on the ends. Before grinding, the capillaries were filled with paraffin to prevent plugging with the grinding compound and scratching of the inside walls.

3. The Respirometer Case.—Complete structural details and all dimension data for the

respirometer case are given in the drawing (Fig. 1). The case is essential for the operation of the respirometer only in that it is necessary to provide some sort of clamp for holding the head plate tightly to the chamber block.

4. Accessories.- Chamber cups for holding tissue were constructed of thin walled glass tubing closed and flattened at one end and cut to the proper length. The bottoms were ground to reduce their thickness.

Mixing cells, for mixing solutions inside the respirometer, were constructed of a glass ring and two glass plates, as shown in the drawing. To complete the mixing cells rings of paraffin were placed on one face of each disc. The outside diameter of each wax ring was slightly less than the inside diameter of the glass ring. The ends of the glass rings were notched to permit gas diffusion.

The most satisfactory magnetic mixing beads were constructed from bits of razor blade, about 1 mm. square, and covered with a thin capsule of glass.

Discs for holding the alkali for CO_2 absorption were cut from alkali resistant filter paper. Small holes punched in the centers of these discs assured pressure equilibrium between the gas in the chamber cups and that above the absorption discs.

An electromagnet for operating the mixing beads was constructed by winding approximately 1200 turns of No. 20, B. & S. cotton-covered copper wire around a core composed of a bundle of soft iron wires, the core being 12 cm. in length and 15 mm. in diameter. It was operated by six dry-cells.

It was possible to use a millimeter scale placed behind the capillary, for observing the movement of the index droplet, but it was more convenient and accurate to use a low power microscope fitted with an ocular micrometer. This arrangement is shown in Fig. 3.

The kerosene used as the index liquid should be free of resin-forming unsaturates, as has been pointed out by Schmitt (8). We found it sufficient to treat the kerosene for several days with concentrated sulfuric acid and then to store it over sodium hydroxide pellets in a stoppered container.

Calibration and Testing

1. Calibration.- The volumes of the chambers, of the capillary posts, and of the cups and mixing cells must be determined with considerable accuracy if accurate measurements are to be made with the respirometer.

The volumes of the chambers were determined by filling them with mercury, squeezing out the excess mercury by putting a glass plate over the chamber, and then pouring the residual mercury into a container for weighing.

The volumes of the capillary posts were calculated from the sizes and lengths of the holes through them.

The volumes of glass in the mixing cells and chamber cups were determined by weighing them and dividing the weight by the density of the glass.

The capillaries were calibrated by taking several measurements of the inside diameter with an ocular micrometer and microscope, and calculating the cross-sectional area. The effective cross-sectional area of the capillary in use, however, is somewhat less than this due to the layer of kerosene adhering to the inner wall. A few experiments were conducted to determine the magnitude of this error. The determinations were carried out in the following manner:

A small droplet of kerosene was introduced into one end of a piece of clean, dry capil-

lary tubing. The length of this droplet was measured with an ocular micrometer. The droplet was then permitted to flow slowly along the tube (by tilting the tube slightly) for a measured distance. The tube was then tilted slightly in the opposite direction. When the droplet had reached its original position its length was again measured. The initial and final volumes of the droplet were calculated. The difference represented the amount of liquid adhering to the walls over the measured distance. This is equal to the error caused by the layer of liquid on the walls of the capillary. The volume of liquid on the wall divided by the total volume of capillary traversed by the droplet $\times$ 100 repre-

FIG. 3. General view of apparatus in use. Microrespirometer below dissecting microscope. The electromagnet is shown above and slightly to the left of the pencil and pad.

sents the percentage error from this cause. The results of eight such experiments are summarized in Table I.

The mean per cent error from this source is 0.45 $\pm$0.10 for the 0.57 mm. diameter capillary, and 0.42 $\pm$0.07 for the 0.22 mm. capillary. These errors are not significantly different for the two sizes of capillary tubing, as determined by the rather approximate method outlined above. The absolute value of the capillary error is not of significant magnitude in most respiration measurements.

2. Testing.—As a final check on the mechanical reliability of the apparatus it was used to measure the volumes of CO_2 liberated from known amounts of standard bicar-

bonate solution by an excess of sulfuric acid solution. The head plate was lightly lubricated with mineral oil and slipped onto the chamber block from the side, using considerable pressure. The respirometer was placed in the case and tightly clamped by turning the screw at the bottom of the case. A capillary containing an index droplet of kerosene was put in place between the capillary posts and sealed at the edges with a little melted paraffin. 10 minutes were allowed for temperature equilibrium. The position of the bubble was then observed with a low power microscope fitted with an ocular micrometer. If the contact surfaces of the chamber block and head plate had been properly ground the position of the bubble did not shift by more than 0.05 mm. during the ensuing 10 minutes. This served to show whether the grinding of these surfaces had been satisfactory.

The head plate was then removed by a sidewise movement and the bottom plates and rings of the mixing cells put in place in the chambers. A measured amount of

TABLE I

Error Resulting from Index Liquid Adhering to the Wall of the Capillary

Experiment No.	Capillary diameter	Error
	mm.	*per cent*
1	0.57	0.44
2	0.57	0.35
3	0.57	0.48
4	0.57	0.55
5	0.22	0.47
6	0.22	0.35
7	0.22	0.43
8	0.22	0.42

standard bicarbonate was put into each mixing cell. A similar amount of sulfuric acid solution of approximately two and one half times the strength of the bicarbonate solution was placed on the bottom surface of each top plate. The mixing bead was put into the bicarbonate solution in one chamber, which will be called "the reaction chamber," and the top plates dropped into place on top of each ring, with the drop of acid projecting downward. The head plate was put on the respirometer and the apparatus set up as previously described. Previous to placing the capillary in position between the posts, it was tilted slightly to shift the kerosene droplet toward the end of the capillary connected to the reaction chamber. When the index droplet had reached equilibrium the acid and bicarbonate in the reaction chamber were mixed by placing the electromagnet above the chamber and drawing the mixing bead from the lower drop to the upper drop, thus bringing the two in contact and mixing the acid and bicarbonate. 10 minutes were allowed for the reaction to go to completion. The position of the index droplet was again observed and from its displacement the volume of CO_2 liberated was calculated, as discussed under the section, "Theoretical Considerations and Calculations." These experiments served as a final check on the calibrations and mechanical features of the respirometer. The results of fourteen such experiments are summarized in Table II.

Respiration Measurements

After calibrating and testing the apparatus as previously described, it was used for a series of measurements of the oxygen consumption of *Paramecium caudatum*, part of which are summarized in Table III.

These experiments were carried out as follows: A measured volume of a suspension of the organisms was placed in one chamber cup and an equal volume of the same medium, free of organisms, was placed in the other. The cups were put in the chambers

TABLE II

Measurement of Volume of CO_2 Displaced from a Known Amount of Bicarbonate by the Addition of Excess Acid

Experiment No.	Volume of CO_2 calculated (λ)	Volume of CO_2 measured (λ)	Error
			per cent
1	2.60	2.37	−7
2	2.60	2.48	−5
3	3.00	2.72	−10
4	3.15	3.12	−3
5	2.98	2.85	−4
6	2.38	2.46	+3
7	2.95	3.01	+2
8	2.95	3.01	+2
9	2.95	3.01	+2
10	2.16	2.09	−3
11	2.16	1.99	−8
12	2.16	2.08	−4
13	2.16	2.08	−4
14	3.42	3.60	+5
Average per cent error			±4.5
Average per cent recovery			98

and an absorption disc put on top of each cup. The same amount (a few λ) of 0.5 N NaOH was placed on each disc. The apparatus was then set up as previously described and the position of the index droplet was recorded at the intervals indicated in Table III. The O_2 consumption values were calculated from these figures. It was possible to continue oxygen consumption measurements indefinitely, since, as the droplet approached one end of the capillary, rotation of the head plate through 180° caused the droplet to move in the opposite direction. The reproducibility of measurements is also shown by the table. The number of organisms used in these experiments varied from 43 to 231.

Theoretical Considerations and Calculations

The theoretical considerations involved in the testing of the apparatus by liberations of CO_2 may conveniently be discussed with the aid of two idealized

TABLE III

Reproducibility of Results in Measuring the Oxygen Consumption of Paramecium caudatum

Experiment No.	Trial No.	O_2 consumption		
		Mm. displacement/min.		λO_2 at standard conditions per hour
1	1	0.30	0.26 ±0.04*	0.66 ±0.10
	2	0.26		
	3	0.25		
	4	0.23		
2	1	0.32	0.33 ±0.02*	0.85 ±0.05
	2	0.35		
3	1	0.20	0.21 ±0.01†	0.54 ±0.02
	2	0.22		
	3	0.22		
4	1	0.30	0.29 ±0.01*	0.74 ±0.02
	2	0.28		
5	1	0.14	0.14 ±0.01†	0.35 ±0.02
	2	0.15		
6	1	0.10	0.09 ±0.01†	0.23 ±0.02
	2	0.08		
7	1	0.53	0.53 ±0.05*	1.35 ±0.13
	2	0.51		
	3	0.58		
	4	0.50		

* Measurements made at 10 minute intervals.

† Measurements made at 20 minute intervals.

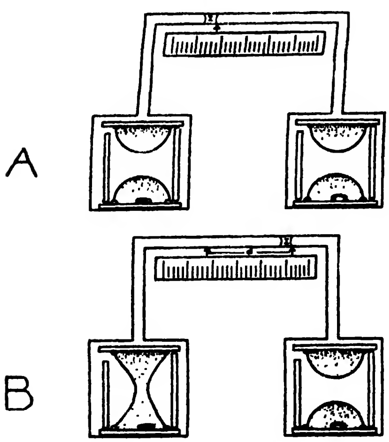

FIG. 4. Illustration of the operation of the apparatus in testing by calibration of CO_2.

diagrams representing the conditions before and after liberations of the gas—Figs. 4 A and 4 B respectively.

The chambers, connected by small openings through capillary posts and capillary constitute a closed system which is separated into two parts by an index droplet *I*. *I* may be regarded as a frictionless piston having a component of motion in the horizontal only, so that at equilibrium the gas pressures at the two ends of *I* are the same.

If, now, gas is liberated in one chamber (by the mixing of acid and bicarbonate in the left-hand chamber as shown in Fig. 4 B), then the pressure in this

chamber rises, forcing the index droplet to the right. This movement compresses the gas in the right-hand chamber. The movement continues until the pressures in the two chambers are the same. Since the displacement of the index droplet is what is measured, it becomes necessary to interpret the observed displacement in terms of volume of gas liberated.

In the discussion of the relationship between observed displacement and volume of gas liberated (or consumed) the following symbols will be used:

1. V_R = volume* of gas in the reaction chamber (the left-hand chamber in Fig. 4). This includes the volume of the chamber proper plus the volume of the capillary up to the index droplet, minus the sum of the volumes of mixing cell, mixing bead, bicarbonate solution, and acid solution.
2. V_C = volume of gas in the compensation chamber.
3. ΔV_{P_0} = volume of gas liberated or consumed measured at P_0 and t.
4. t = temperature of apparatus in degrees Centigrade.
5. Vl_R = volume of liquid in the reaction chamber.
6. Vl_C = volume of liquid in the compensation chamber.
7. P_0 = initial pressure in the system as shown in Fig. 4 A, = barometric pressure at the time the apparatus is set up.
8. P_f = final pressure in the system, as shown in Fig. 4 B.
9. d = observed displacement in millimeters of the index droplet.
10. A = effective cross-sectional area of the capillary in mm.^{2} = measured area minus 0.4 per cent.
11. αCO_{2_t} = the solubility coefficient of CO_2 at temperature t and 760 mm. pressure of CO_2, in terms of volumes of gas at standard conditions per volume of liquid.
12. αN_{2_t} = ditto for N_2.
13. αO_{2_t} = ditto for O_2.

* All volumes are expressed in λ (mm.^{3}).

It is clear that if the index droplet I has been observed to move d mm., V_C has been diminished by $Ad\lambda$. The pressure in the compensation chamber has therefore increased from P_0 to $\left(\frac{V_C}{V_C - Ad}\right)P_0$. We shall call this higher pressure P_f. It is equally clear that the pressure in the entire system now has this same value of P_f, since the pressures at the two ends of I are the same. Therefore a gas occupying a volume of $(V_R + V_C)$ at a pressure P_0, now occupies this same volume at a pressure P_f. $(V_R + V_C)$ at P_f would be $(V_R + V_C)\frac{P_f}{P_0}$ at P_0. In other words, if the liberated gas had been allowed to expand freely against a pressure P_0 the total volume of gas would have been increased by $(V_R + V_C)\frac{P_f}{P_0} - (V_R + V_C)$. We shall call this increase ΔV_{P_0}. ΔV_{P_0} represents the volume of gas liberated from

the bicarbonate solution. It becomes necessary then to determine the relation between d and ΔV_{P_0}.

Suppose the ratio of the total volume of the two chambers to the volume of the compensation chamber is n,

$$V_C = \frac{V_B + V_C}{n}$$

then

$$nV_C = V_B + V_C$$

since

$$\Delta V_{P_0} = (V_B + V_C)\frac{P_f}{P_0} - (V_B + V_C)$$

$$\Delta V_{P_0} = nV_C\frac{P_f}{P_0} - nV_C$$

but

$$P_f = \left(\frac{V_C}{V_C - Ad}\right)P_0$$

therefore

$$\Delta V_{P_0} = nV_C\left(\frac{V_C}{V_C - Ad} - 1\right)$$

or

$$\Delta V_{P_0} = nAd\left(\frac{V_C}{V_C - Ad}\right) \tag{1}$$

Since Ad is usually small with respect to V_C the expression: $\left(\frac{V_C}{V_C - Ad}\right)$ is approximately equal to one and equation (1) reduces to

$$\Delta V_{P_0} = nAd \tag{2}$$

ΔV_{P_0}, calculated from d by means of either (1) or (2) may be reduced to standard conditions in the customary manner.

ΔV_{P_0} however, does not represent all the CO_2 produced from the bicarbonate, since an appreciable fraction of the CO_2 remains dissolved in the liquid in the reaction chamber. The quantity of dissolved CO_2 may be calculated, since it is known that a volume of CO_2, ΔV_{P_0}, permeates a space, $(V_B + Ad)$ in which the total gas pressure is equal to Pf. The partial pressure of CO_2 in this space is therefore equal to $[\Delta V_{P_0}/(V_B + Ad)]\ Pf$. If the temperature of the apparatus is t, then the quantity of dissolved CO_2 is given by the formula:

$$CO_2\text{ (dissolved)} = \left(\frac{\Delta V_{P_0}}{V_B + Ad}\right) Pf\,\alpha CO_{2_t} \cdot Vl_B \tag{3}$$

Since αCO_2 is given in terms of volumes of gas at standard conditions per volume of liquid, it is only necessary to add the reduced value of ΔV_{P_0} to the volume of dissolved CO_2 in order to get the total volume of CO_2 produced by the reaction.

For measurements of greater precision it is necessary to take into consideration an additional source of error. It is apparent that the compression of the gas in the compensation chamber has increased the partial pressure of O_2 and N_2 in this chamber and that more of these gases have gone into solution. At the same time the partial pressures of oxygen and nitrogen have diminished in the reaction chamber, since the same quantities of these gases now occupy a larger volume. There is a smaller volume of dissolved oxygen and nitrogen in the reaction chamber and a greater volume of dissolved oxygen and nitrogen in the compensation chamber than was initially the case. This produces a greater displacement of the index droplet than if the liquid phase were absent.

The partial pressures of oxygen and nitrogen in the compensation chamber have increased to P_f/P_0 times their original values. The partial pressures of oxygen and nitrogen in the reaction chamber have decreased to $\frac{V_R - Ad}{V_R}$ times their original values.

The additional quantity of oxygen forced into solution by compression in the compensation chamber is equal to:

$$\Delta O_2 \text{ (dissolved)} = \alpha O_{2t} \cdot Vl_C \cdot P_{O_2} \cdot \left(\frac{P_f}{P_0} - 1\right) \tag{4a}$$

where P_{O_2} = initial partial pressure of oxygen.

Similarly for N_2

$$\Delta N_2 \text{ (dissolved)} = \alpha N_{2t} \cdot Vl_C \cdot P_{N_2} \cdot \left(\frac{P_f}{P_0} - 1\right) \tag{4b}$$

The quantities of oxygen and nitrogen released from solution in the reaction chamber are:

$$\Delta O_2 \text{ (dissolved)} = \alpha O_{2t} \cdot Vl_R \cdot P_{O_2} \cdot \left(\frac{VR - Ad}{VR} - 1\right) \tag{4c}$$

$$\Delta N_2 \text{ (dissolved)} = \alpha N_{2t} \cdot Vl_R \cdot P_{N_2} \cdot \left(\frac{VR - Ad}{VR} - 1\right) \tag{4d}$$

The latter two quantities are negative, but since they tend to produce an error in the same direction as the first two, all four Δ's are added, and the sum subtracted from the reduced volume of CO_2 already calculated.

Respiration Measurements

The principles involved in respiration measurements are the same as those already discussed. In this case, however, we have to deal with a system in which the total pressure is decreasing.

$$\Delta V_{P_0} \text{ is therefore equal to: } nAd\left(\frac{V_C}{V_C + Ad}\right) \tag{5}$$

A source of error in respiration measurements is introduced by the removal of only one component (oxygen) of the gas mixture in the reaction (respiration) chamber. The partial pressures of oxygen and nitrogen do not remain equal in the two chambers. If the displacement volume $= Ad$, V_C is increased to $V_C + Ad$. The partial pressures of oxygen and nitrogen in the compensation chamber are thus reduced to $\frac{V_C}{V_C + Ad}$ times their initial value.

In the reaction chamber the volume has been reduced to $V_R - Ad$. The partial pressure of nitrogen in the reaction chamber has therefore increased to $\left(\frac{V_R}{V_R - Ad}\right)$ times its initial value. The partial pressure of oxygen, on the other hand, has diminished to:

$$\frac{V_R}{V_R - Ad + nAd} P_{O_2} \quad \text{or} \quad \frac{V_R}{V_R - Ad(n - 1)} P_{O_2} \tag{6}$$

The changes in the volumes of dissolved oxygen and nitrogen may then be calculated as already outlined and the correction applied accordingly.

In calculating the initial partial pressures of oxygen and nitrogen, it should be remembered that water vapor constitutes an appreciable fraction of the gas mixture in the chambers.

SUMMARY

1. A microrespirometer suitable for measuring oxygen uptakes from 0.1 to 10λ per hour is described.

2. The sensitivity of the instrument may be readily altered by substituting different sizes of capillary tubing.

3. By means of replaceable brass plugs the chamber volume of this instrument may be varied from 700 to less than 40λ.

4. No thermostat is required for the operation of the instrument at room temperature.

5. It may be charged at one temperature and used at a widely different one.

6. The chambers may be filled with any desired gas mixture.

7. Two solutions may be mixed during the course of an experiment.

8. The entire apparatus may be sterilized.

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BIBLIOGRAPHY

1. Barcroft, J., *J. Physiol.*, 1908, **37**, 12.
2. Barcroft, J., Respiratory function of the blood, Cambridge University Press, 1914.
3. Dixon, M., Manometric methods, Cambridge University Press, 1934.
4. Duryee, W. R., *Z. vergleich. Physiol.*, 1926, **23**, 208.
5. Fenn, W. O., *Am. J. Physiol.*, 1927, **80**, 327.
6. Fenn, W. O., *Am. J. Physiol.*, 1928, **84**, 110.
7. Heatley, N. G., Berenblum, I., and Chain, E., *Biochem. J.*, London, 1939, **33**, 1.
8. Schmitt, F. O., *Am. J. Physiol.*, 1932, **104**, 303.
9. Thunberg, T., *Skand. Arch. Physiol.*, 1905, **17**, 74.
10. Victor, J., *Am. J. Physiol.*, 1935, **111**, 477.

THE UPTAKE* OF INORGANIC ELECTROLYTES BY THE CRAYFISH†

BY N. S. RUSTUM MALUF

(*From the Department of Zoology, Johns Hopkins University, Baltimore, Maryland, and the U. S. Fish Commission, Woods Hole, Massachusetts*)

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INTRODUCTION

Ever since the latter part of the nineteenth century botanists have realized that plants are capable of accumulating minerals from fresh water, against concentration gradients, and have more recently made first attempts at unravelling the mechanism of the process (see for instance Hoagland, 1923; Hibbard *et al.*, 1926; Steward, 1933; Jacques and Osterhout, 1935; Rosenfels, 1935; Lundegårdh, 1937 and earlier). As far as the writer is aware, the first demonstration of such a kind in a fresh water member of the animal kingdom was made by Schumann (1928) who, in an apparently little read paper, showed that the newly molted amphipod crustacean, *Gammarus pulex*, can absorb Ca from very dilute solutions (1 to 10 mEq./l.). It is sometimes assumed that molluscs take up Ca^{++} from fresh water in the calcification of their shell. As far as is known, this has never been actually demonstrated and it may be just as likely that they accumulate Ca ingested with the food. Considerable preliminary work on the uptake of electrolytes by animals initiated by Koch and Krogh (1936), has recently been reviewed by Krogh (1939). In forms living in brackish water, with a saline concentration in the blood markedly higher than that of the environment, the phenomenon has been shown to occur in the shore crab, *Carcinus maenas*, by Nagel (1934).

The crayfish (*Cambarus clarkii* in this work) appears admirably suited for studies on the mechanisms of this uptake since it is hardy, can with-

* The word "uptake," as used here, implies the transport of a solute against its concentration gradient.

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stand several months without food (Brunow, 1911), exhibits a powerful capacity for Cl uptake as compared with other fresh water animals (Krogh, 1939), is of moderate size, readily obtained, and inexpensive.

The concentration of the various ions (Na^+, K^+, Ca^{++}, Cl^-, $SO_4^=$) in the external solutions was about 7.5 mEq./l. Their approximate milliequivalence per liter in the blood of the crayfish is 194 for Cl, 135 for Na, 14.5 for K, and 16 for Ca^{++}. The above figures for *Potamobius astacus* (the European crayfish) were obtained from the data of Griffiths (1891) and Huf (1935) since they balance up better than do those of Lienemann (1938) for *C. clarkii* and do not have the abnormally low K value (= 3.1 mEq./l.) of Bogucki (1934). The data, as a whole, indicate that the concentrations of the various inorganic electrolytes in the blood of *P. astacus* and *C. clarkii* are nearly identical.

The crayfish were put in pure dilute solutions of NaCl, KCl, Na_2SO_4, or $CaCl_2$. The attempt was made to ascertain whether the cation and anion of a given electrolyte are absorbed independently of each other and, if so, which ions are absorbed and which left behind. Since, at the outset, no Ca^{++} absorption could be detected the question arose as to whether Ca^{++} is absorbed during hardening of the new cuticle at the molting period. An attempt is also made to evaluate the hypothesis that the uptake of Cl might be of significance in the discharge of respiratory CO_2. Evidence for the anatomical site(s) of the uptake is presented. This paper can be regarded only as a preliminary step in the solution of the mechanisms of the uptake of inorganic electrolytes by animals.

Material

The crayfish were shipped by railroad from New Orleans by the Southern Biological Supply Company, in damp *Sphagnum* moss in batches of about 35 and were received 2 days following. They were immediately put into a slanting wooden tank perforated at such a level as to permit some walking space free from the continuously renewed aerated tap water. Since crayfish can live for a few months without food, the animals were not fed and a stock was completely utilized within a month or two. Only vigorous individuals were used in the experiments.

RESULTS

1. *Site of Uptake of the Electrolytes.*—The site of active uptake of electrolytes, present in the body fluids of an animal, is commonly granted to the gills. This has been demonstrated for the anal gills of fresh water dipterous larvae by Koch and Krogh (1936). Koch (1934) found that the anal gills of the *living* larvae and the internal face of the branchiostegites of *living*

prawns will accumulate Ag if put into 0.05–0.1 per cent $AgNO_3$ for a short time and was the first to suggest that the main function of the anal gills is the transport of ions against concentration gradients.

If a living crayfish was briefly rinsed with distilled water and put into 0.025 per cent $AgNO_3$ in distilled water in the dark it died in a little over an hour and, if subsequently put into 10 per cent formalin in the light with branchiostegites removed, to aid reduction of the silver, within a few days the gill stems and gill filaments acquired a dark brown color with a metallic sheen when seen in strong light. Other structures did not behave thus even though the individual used had but lightly pigmented pleopods and venter so that changes in their color could be readily noticed. Paraffin sections were made of the gills; the only stain used was eosin. It was found that the dull brown impregnations occurred only in the cuticle (Fig. 1, *C*) and were easily distinguished from the sparsely scattered bright yellowish brown melanin pigment of the cuticle. The hypodermis, *H*, was never impregnated. The experiment was repeated with similar results. It appears that the Ag impregnation was a veritable accumulation and not merely a diffusion since an animal placed in the Ag solution about 3 hours after it had been killed by decerebration did not absorb Ag.

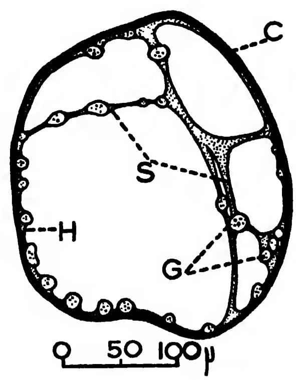

FIG. 1. Transverse section through the gill filament of an 18 gm. crayfish (*Cambarus clarkii*). *C*, cuticle; *G*, "branchial kidneys" of Cuénot; *S*, hypodermal strands separating the efferent from the afferent blood sinuses; *H*, hypodermis—a syncytium.

The uptake of inorganic electrolytes by a fasting animal does not occur by way of the alimentary tract. Enough phenol red was dissolved in the tap water to give an intense red. Even a very small drop of the water could be rendered an intense red by ammoniacal vapor. Two animals were fully immersed in such a solution and in 6 hours the kidneys, blood, alimentary tract, and the hepatopancreas were examined for phenol red by means of ammonia. The results were utterly negative and demonstrate that a crayfish does not ordinarily drink (for further evidence, see Maluf, 1937 *a*). By the same method, it was found that, in a hypertonic medium, such as 0.2 or 1.2 M NaCl, the crayfish does swallow some water; a probable explanation for this has been offered (Maluf, 1937 *a*).

The above results and the fact that, in the absorption experiments below, the medium in which the crayfish was immersed did not extend above the lower margins of the branchiostegites (the medium being pumped into the

gill chambers by the scaphognathites) make it very probable that, apart from Ca^{++} (see below), the electrolytes are taken up by the gill stems and gill filaments.

2. Rôle of the Uptake of Electrolytes. (*a*) *Replenishment for Saline Loss.*—There can be no doubt that the retention of Cl by the gills and the absorption of the same is an active process from the mere fact that a recently dead animal, even with mouth and anus plugged, loses Cl at a very marked rate while living controls from the same stock absorb the same. When an animal absorbs Cl from fresh water in which the Cl concentration is 0.197–0.216 mEq./l., while the Cl concentration of its blood is about 180 mEq./l., it appears pertinent to suppose that the process is of use to the animal. In some cases, the Cl concentration in 200 cc. of fresh water was diminished to such an extent by a 10 gm. animal within 24 hours as to yield no titrable value with the micro-methods below (*i.e.* was probably below 0.15μM Cl) and the $AgNO_3$ test was only barely positive.

A crayfish in fresh water can compensate entirely for Cl lost in the urine by active Cl absorption. Thus, the daily rate of urine production by *C. clarkii* when fully immersed in fresh water is about 2.5 per cent of the wet weight (Lienemann, 1938). The Cl concentration of the urine can attain about 10 mEq./l. under normal conditions (Lienemann). The latter value is doubtless high since a correction was not made for the notable amount of water lost from the urine during the suction process of urine collection. Taking it at face value, however, a 10 gm. crayfish may lose, by way of its kidneys, approximately 0.038 mEq. Cl per 24 hours. A partially starved animal, with both excretory orifices sealed, can show a rate of Cl absorption from fresh water (containing about 0.2 mEq. Cl/l.) of at least 0.069 mEq./10 gm./20 hours. In higher, but nevertheless blood-inferior, Cl concentrations the rate of Cl uptake is greater (Table I).

Since Cl is not stored by tissues as far as is known, one would assume that a crayfish, in order to exhibit Cl absorption which is many times above that which could conceivably result from a mere storage of urine in the bladders, must have undergone Cl depletion. The crayfish, however, even on immediate receipt at all times of the year from the Southern Biological Supply Company, invariably displayed the capacity for Cl absorption. The reason for this may have been due to deficient dieting before shipment. The Supply Company states that "these crayfish were brought in by our collectors and placed in tanks without food about two days prior to shipment to you. . . . They had been placed in approximately two to three inches of city tap water in a large tank, one part of which was dry in order to enable the crayfish to come out and aerate themselves." Nothing

TABLE I

Uptake of Inorganic Electrolytes by the Crayfish

Crayfish (identical nos. refer to identical individuals)	Weight in gm. and sex	Duration of experiment	Type of solution	Initial concn. in mEq./l.	mEq. absorbed per 10 gm. per 20 hrs.					Date
					Cl	Na	K	Ca	SO_4	
		hrs.								
1	14.4♂	14.2	NaCl	7.35	0.234	0.202	—	—	—	Dec. 10–11
2	18.8♀				0.075	0.172	—	—	—	
1*a*	14.3♀	19.0	NaCl	7.25	0.066	0.0735	—	—	—	Dec. 31–Jan. 1
2*a*	11.7♀				0.117	0.090	—	—	—	
1	(As above)	14.5	KCl	7.60	0.00	—	0.00	—	—	Jan. 8–9
2	" "				0.02	—	0.00	—	—	
1	" "	17.0	NaCl	7.80	0.164	—	—	—	—	Jan. 11–12
2	" "				0.100	—	—	—	—	
1	" "	16.3	KCl	8.30	0.020	—	—	—	—	Jan. 12–13
2	" "				0.00	—	—	—	—	
1*a*	" "	18.5	KCl	8.10	0.106	—	—	—	—	Jan. 13–14
2*a*	" "				0.018	—	—	—	—	
1*a*	" "	17.0	NaCl	7.90	0.105	—	—	—	—	Jan. 14–15
2*a*	" "				0.166	—	—	—	—	
1*a*	" "	18.0	KCl	7.80	0.031	—	0.00	—	—	Jan. 16–17
14	27.1♂	20.0	KCl	7.80	0.059	—	0.00	—	—	Jan. 18–19
15	19.4♀				0.072	—	−0.010 (a loss)	—	—	
16	29.5♂				0.027	—	−0.017 (a loss)	—	—	
14	(As above)	24	NaCl	8.40	0.0675	0.077	—	—	—	Jan. 22–23
15	" "				0.111	0.159	—	—	—	
16	" "				0.113	0.158	—	—	—	
14	" "	20.7	Na_2SO_4	7.35	—	0.0356	—	—	0.000	Feb. 14–15
15	" "				—	0.0772	—	—	0.000	
16	" "				—	0.0687	—	—	0.000	
14	" "	23.5	Na_2SO_4	7.40	—	0.0502	—	—	0.000	Mar. 2–3
15	" "				—	0.0345	—	—	0.000	
2 (hard-shell)	" "	23.0	$CaCl_2$	7.40	0.037	—	—	0.000	—	Mar. 15–16
x (molted Mar. 7)	8.3♂				0.105	—	—	0.42	—	
30 (hard-shell)	11.1♂	22.0	$CaCl_2$	7.40	0.000	—	—	−0.016 (a loss)	—	Mar. 18–19
x	(As above)				0.000	—	—	0.284	—	
40 (preparing to molt; gastroliths fully developed)	13.5♂				0.000	—	—	0.188	—	
32 (hard-shell)	14.5	24.0	$CaCl_2$	7.20	0.0575	—	—	0.000	—	Mar. 22–23
m (molted late in Dec., kept in tap water)	9.3				0.0537	—	—	0.000	—	
42 (hard-shell)	9.4				0.0354	—	—	0.000	—	
1*c*	9.0	12.7	NaCl	7.68	0.256	—	—	—	—	Aug. 26–27
2*c*	9.25		NaCl	7.68	0.243	—	—	—	—	
3*c*	8.0		NaCl	7.68	0.270	—	—	—	—	
4*c*	8.2		$CaCl_2$	14.9	0.173	—	—	—	—	
5*c*	7.0		$CaCl_2$	14.9	0.025	—	—	—	—	
1*d*	8.0	13.2	NaCl	8.02	0.208	—	—	—	—	Aug. 27–28
2*d*	9.0		NaCl	8.02	0.171	—	—	—	—	
3*d*	10.4		NaCl	8.02	0.160	—	—	—	—	
4*d*	7.0		$CaCl_2$	8.45	0.00	—	—	—	—	
5*d*	8.6		$CaCl_2$	8.45	0.104	—	—	—	—	
6*d*	7.3		$CaCl_2$	8.45	0.122	—	—	—	—	
1*d*	(As above)	12.0	$CaCl_2$	8.45	0.00	—	—	—	—	Aug. 29–30
2*d*	" "		$CaCl_2$	8.45	0.144	—	—	—	—	
3*d*	" "		$CaCl_2$	8.45	0.104	—	—	—	—	
4*d*	" "		NaCl	8.02	0.087	—	—	—	—	
5*d*	" "		NaCl	8.02	0.115	—	—	—	—	
6*d*	" "		NaCl	8.02	0.206	—	—	—	—	

was known, apparently, of the state of nutrition of the animals upon capture.

(*b*) *Release of Respiratory CO_2.*—In 1937 Ferguson, Lewis, and Smith noted that the activity of carbonic anhydrase in various marine invertebrates is greatest in the gills, less in the muscles, and practically nil in the blood. They stated that this enzyme may be of importance in the discharge of CO_2 by the gills but nevertheless pointed out that, if such is the case, it would be "hard to see how bicarbonate ions could enter the cells of the gill to be changed to CO_2, except by exchange with some anion of the gill cell, *e.g.* chloride ion. . . . To make such a scheme acceptable a mechanism for replenishing the chlorides in the gill cells would have to be found." Lundegårdh (1933, 1937) has noted that the ratio of the rate of CO_2 evolution to anion absorption is approximately constant in the rootlets of healthy barley plants; that this ratio differs with different anions (Cl^-, NO_3^-, $SO_4^=$); and that the absorption is continuous. On the other hand, in frogs and fresh water fishes (*i.e.* in erythrocyte-possessing animals), Krogh (1937 *a*, *b*) has found that Cl absorption is not continuous but only follows Cl depletion of the tissues. In such animals, then, Cl absorption cannot be of respiratory significance.

A comparison of the rates of CO_2 output and of Cl uptake by the crayfish (*Cambarus clarkii*) courts the apparently inevitable conclusion that Cl absorption is of no respiratory significance in this animal since the rate of CO_2 output (= *ca.* 3.0 mEq. CO_2/10 gm./20 hrs. at 25°C., according to Maluf, 1937 *b*) is many times in excess of the net rate of Cl absorption from fresh water even by the partially starved animal (= *ca.* 0.069 mEq. Cl/10 gm./20 hrs. at 25°C.). Furthermore, the Cl-absorbing capacity of the crayfish (*Potamobius astacus*) has been noted by Krogh (1939) to be "very powerful" compared with that of other fresh water animals; it therefore seems most unlikely that Cl uptake is of any respiratory significance in animals.

It was nevertheless considered of interest to measure the carbonic anhydrase activity of the gills of the crayfish and to compare this activity with that of other tissues of the same animal. The measurements were made at Woods Hole with the same apparatus used by Ferguson *et al.* The activity, *E*, is expressed per gram of fresh tissue at 15°C. at 50 per cent completion of the reaction. The tissues were pooled from six healthy animals.

The carbonic anhydrase activity of the gills, in the crayfish too, is markedly greater than that of other tissues. The blood of this invertebrate, however, shows a definite carbonic anhydrase activity.

Crayfish
(*Carbonic Anhydrase Activity*)

	Gills	Whole blood	Hepatopancreas	Integument (hard-cuticle)	Abdominal somatic muscles
E	80	26	17	15	15

Since carbonic anhydrase will accelerate any reaction in which the formation or decomposition of carbonic acid is the limiting reaction (Meldrum and Roughton, 1933) and since it may thus be of importance in the deposition and dissolution of $CaCO_3$ in the cuticle[1] of Crustacea, measurements were made of the carbonic anhydrase activity of the integument of the lobster (*Homarus americanus*) at pre-molt, post-molt, and hard-cuticle periods and upon other tissues of the lobster for comparison. As the following table shows, the hypodermis of the hard integument has practically the same carbonic anhydrase activity as the new integument shortly prior to molting and as the soft parts of the hardening integument following molting by about 1 week. All the activities are relatively low. The integumental samples were obtained from the branchiostegites where they are practically free from extraneous tissue.

Lobster
(*Carbonic Anhydrase Activity*)

	Gills	Abdominal somatic muscles	New integument prior to molt	Soft part of hardening integument 1 week after molt	Hypodermis of hard integument	Crop tissue
E	19–22	20	19	17	16	17

3. Manner of Uptake of the Electrolytes from Blood-Inferior Solutions.—It will be noted from Table I (Dec. 10–11, Dec. 31–Jan. 1, Jan. 22–23) that, in pure NaCl, Na^+ and Cl^- are not absorbed at strictly identical rates. The correspondence is quite close but the differences exceed the limits of error. In pure KCl, K^+ is not taken up at all while the rate of Cl^- uptake, by the same individuals, is generally lower than that in NaCl of approximately the same normality (Jan. 8–9, Jan. 11–12, Jan. 12–13, Jan. 13–14, Jan. 14–15, Jan. 16–17, Jan. 18–19, Jan. 22–23). This indicates that NaCl is absorbed, partly at least, in molecular form. The results also show that Cl^- can be taken up independently of the cation. In the intervals between such experiments involving back-and-forth transfer between two different

[1] The cuticle is the entire non-living component of the integument.

solutions, the animals were returned to individual battery jars containing distilled water.

In pure Na_2SO_4, the $SO_4^=$ is left behind entirely (Feb. 14–15, Mar. 2–3) while the Na^+ is absorbed at a rate lower than that from the same normality of NaCl (compare Jan. 22–23 with Feb. 14–15) even though the same animals were subjected to distilled water in separate jars for over 3 weeks between the NaCl and Na_2SO_4 runs. These experiments show that the Na^+ can be absorbed independently of the anion and indicate that NaCl is, to a large extent, taken up as a whole molecule; which, latter, is a confirmation of an identical statement in the previous paragraph.

From pure $CaCl_2$, a hard-cuticled crayfish not preparing to molt will leave the Ca^{++} behind and generally absorb some Cl^- (Mar. 15–16, Mar. 18–19, Mar. 22–23, Aug. 26–30). An animal which has recently molted or which is preparing to molt will absorb Ca^{++} but not necessarily the Cl^- complement (Mar. 15–16, Mar. 18–19). This demonstrates that Ca^{++} can be taken up independently of the anion and that, unlike conditions with NaCl, the Ca^{++} and Cl^- are not absorbed together. The rate of Cl absorption, by the same individuals, from $CaCl_2$ is notably less than from NaCl of about the same normality (Aug. 26–30, Mar. 15–23), which corroborates the above statements that Na^+ and Cl^- are absorbed largely as NaCl. The periodic uptake of Ca^{++} during specified conditions, the unusually high rate of its uptake as compared with Na^+ or Cl^- when taken alone, and the evidence that, unlike Na^+, it is not absorbed together with the Cl^- component, all strongly suggest that Ca^{++} is probably not absorbed through the gills but deposited directly in the cuticle, as $CaCO_3$, presumably through the agency of some enzyme.

4. *Active Uptake of Calcium and the Hardening of the Integument after Molting.*—It was shown in the previous section that a newly molted crayfish will take up Ca^{++} at a rate higher than that of the other ions absorbed (Na^+ and Cl^-) and that a hard-cuticled animal will not absorb any appreciable quantity of Ca^{++} unless it is preparing to molt. It appears that a large fraction of the Ca required for the new cuticle is furnished in this way. The Ca^{++} concentration of fresh water bodies may be of importance in determining the existence of crayfish in such. After the above results were obtained, Dr. John H. Lochhead kindly called my attention to Schumann's (1928) paper in which it is shown that newly molted *Gammarus pulex*, a fresh water amphipod, absorbs Ca from concentrations as low as 1 to 10 mEq./l. and that such an animal cannot develop and breed in bodies of water with a Ca concentration lower than 0.26 mEq./l. The "hard"-

cuticled *G. pulex* does not absorb Ca. These results are in accord with those here presented for the crayfish.

One of the few crayfish which molted in the laboratory was placed in distilled water a few hours after molting. Following the molt it was lying on its side apparently incapable of normal posture; the pincers were soft and immobile; the cuticle was so soft that when the animal was lifted up its limbs fell limply; the limb segments were readily pliable; it was capable of flipping its abdomen vigorously when touched; the scaphognathites beat rhythmically; and the chelipeds, walking legs, antennae, and eyes moved frequently and 'spontaneously'. Its weight, added to that of the exuvium, showed that it had absorbed water to the extent of about 51.5 per cent of its body weight as measured 3 weeks before the molt. The animal had not been fed and showed no appreciable increase in its dimensions. In 24 hours the claws were capable of movement and hardening of the cuticle had progressed slightly. The animal attempted to assume the upright posture every now and then. In about 55 hours the upright position was held indefinitely. The cuticle appeared to undergo no further hardening even after the lapse of 2 months at which time the animal died without apparent cause. The per cent raw ash in the cuticle of the carapace of this animal was only 25.6 of the dry weight as compared with 40 or more for a hard-cuticled animal.

Another individual was kept in tap water after molting and showed a definitely more progressive picture, as regards hardening of the cuticle within a given time interval, than the above animal which was not permitted any foreign source of Ca after molting. Distilled water does not appear to have any directly deleterious effect on crayfish since they are capable of living in it indefinitely; it therefore seems most probable that the results here described are due merely to the absence of Ca from the distilled water. These data are necessarily meager but suggestive.

It is appropriate to know how much of the raw ash and organic material in the old cuticle is resorbed during molting, more especially since such data apparently have not hitherto been available for any fresh water crustacean. Knowledge concerning this was made possible partly by the fact that a crayfish was weighed a few weeks before molting; hence its normal weight was known, since a fasting crayfish decreases in weight only very slowly with time. The exuvium of the animal was dried and ashed. To obtain further pertinent data, hard-cuticled crayfish were dried, weighed, and ashed. The integument of a hard-cuticled crayfish, not preparing to molt and with a heavily melanized abdominal venter, was scrupulously

freed of all adherent material including hypodermis. The limbs were incised longitudinally and their insides scraped free of tissue. From such a preparation the raw ash and organic material (by difference) fractions were obtained. Other crayfish were dried and the per cent dry weight, of the total weight, obtained. The data are summarized as follows:

Total weight of crayfish a few weeks before molting	17.5 gm.
Per cent dry weight of total weight	26.0
∴ Total dry weight	4.55 gm.
Per cent dry cuticle of total dry weight	80.4
∴ Weight of cuticle	3.66 gm.
Per cent ash (chiefly CaO) in the cuticle	37.8 gm.
∴ Weight of ash in cuticle	1.38 gm.
But weight of ash in the exuvium of the 17.5 gm. animal	1.325 gm.
∴ Amount of raw ash resorbed	0.055 gm., or *3.98* per cent of the original.
Per cent organic material in the dry cuticle	62.2
∴ Amount of organic material in dry cuticle	2.28 gm.
But weight of organic material in the exuvium of the 1.75 gm. animal	2.228 gm.
∴ Amount of organic material resorbed	0.052 gm., or *2.28* per cent of the initial.

The fact that a fasting crayfish does not increase appreciably in dimensions following molting, in itself indicates that shedding involves an unnecessary loss. The fact that only about 4 per cent of the raw ash of the old cuticle is resorbed and that the animal loses about 84 per cent of the total amount of ash in the body, by far the chief mineral of which is Ca, corroborates such a view.

Methods

The crayfish was wrapped in a dry towel and left thus for a few minutes to remove excess moisture. The water in the branchial chambers and various grooves of the body was then drained by tap suction and the animal, including its branchial chambers, was irrigated with the solution like that (*i.e.* from the same stock) in which it was to be immersed. It was then put into a 15 × 7.5 cm. crystallizing dish, containing 200 cc. of the solution, with the cover supported by a 0.5 cm. (diam.) glass rod across the mouth. The solution in the dish did not extend far above the lower margins of the branchiostegites but it was deep enough to be pumped into the branchial chambers by the scaphognathites *ad lib.* Identical crystallizing dishes without crayfish were maintained as controls. The room temperature varied from 24–27°C. In the later experiments it was not considered necessary to plug the excretory orifices (see Maluf, 1939)

or drain the bladders before introducing the animals into the solutions inasmuch as it can be shown that the maximum amount of urine that can be stored in both bladders, of a 10 gm. animal for example, contains no more than *ca.* 0.0023 mEq. Cl and much less Ca^{++} and K. Unlike *C. bartoni* (Maluf, 1939), however, Cl absorption by *C. clarkii* does not seem to be notably affected by the dental plugging cement.

Chemical Analyses.—All the methods involved the use of a Linderstrøm-Lang-Keys microburette. This was of 250 c.mm. capacity, divided into cubic millimeters, and of uniform bore as shown by measurements of the length of a drop of mercury at all levels.

Standard solutions of three different concentrations of each electrolyte, closely surrounding the encountered range, were made up and the value of an unknown ascertained by interpolation. All analyses were made in duplicate or triplicate.

Chlorin.—To 1 cc. of the medium in a 9 × 75 mm. test-tube was added 0.5 cc. *ca.* 0.04 N $AgNO_3$ in 68–70 per cent HNO_3; the test-tubes were capped with glass bulbs and heated in boiling water for 15 minutes; 0.1 cc. ferric ammonium sulfate was used as indicator and 1 cc. of pure ethyl alcohol was added to enhance flocculation of the AgCl. Titration was with *ca.* 0.1 N NH_4SCN. For the analysis of Cl in fresh water the same volumes of reagents were used as stated in a previous paper (Maluf, 1939). The accuracy, as shown by triplicates and recovery from standard solutions, was within 1 per cent.

Sodium plus Potassium was measured by Krogh's (1938) modification of Linderstrøm-Lang's (1936) technique. Tamworth "Glasbake" centrifuge tubes (Tamworth Associates, Inc., Needham Heights, Mass.) were substituted for the "supremax" glassware. To remove the NH_4Cl and $(NH_4)_2CO_3$ formed during the procedure, the centrifuge tubes were ignited at 330–360°C. for 2 hours. The aliquots from the supernatant liquids were drawn by means of self-improvised carefully calibrated pipettes of the constriction type with fine tips. The accuracy was within 2 or 3 per cent.

Calcium.—To 1 cc. of the *ca.* 7.5 mEq./l. solution in a 15 cc. "Glasbake" centrifuge tube was added 0.5 cc. concentrated NH_4OH and the whole heated at 50–60°C. in a water-bath for 5 minutes while stirred with CO_2-free air. 1 cc. 0.2 N NH_4 oxalate was added; the tube was stoppered and allowed to stand overnight. Wang's (1935) method of washing was adopted except that, in our relatively pure solutions, only one washing was considered necessary. Ignition was according to the method of Siwe (1935). The residue was completely dissolved, with the aid of a fine glass rod, in 1.4 cc. 0.015 N HCl and the excess acid titrated with 0.1 N NaOH to a weak red with phenolphthalein as indicator. The air current for stirring had been rendered CO_2-free. The accuracy was within 3 per cent.

Sulfate was measured essentially according to Øllgaard's (1934) technique adapted to suit the larger quantities used in this work. To a 2 cc. sample of the *ca.* 7.5 mEq./l. solution in a 15 cc. pyrex centrifuge tube was added 0.1 cc. 99.5 per cent glacial acetic acid. After mixing, 5 cc. of freshly made 1 per cent benzidin in sulfate-free acetone were added and the whole mixed and allowed to stand for about an hour. The tubes were then centrifuged at about 2,000 R.P.M. for 5–6 minutes; the supernatant liquid was decanted as completely as possible without disturbing the residue. 10 cc. sulfate-free acetone were added to the residue without expressly breaking up the latter and the tubes centrifuged at about 2,000 R.P.M. for 5–6 minutes; the supernatant liquid was decanted and the adherent acetone allowed to evaporate. To each residue was added 0.35 cc. *ca.* 0.1 N carbonate-free NaOH. Each tube was then individually immersed in a bath at 75–80°C. and the residue and white particles on the lower wall of the tube

completely dissolved by means of a very fine glass rod. Without permitting cooling to the extent of solidification of the formed benzidin hydrate, the tube was placed in a 250 cc. beaker containing water at about 70°C. and 4 cc. of the alcoholic ammoniacal solution added. The liquid remained *clear* and colorless. Titration, while still in the warm water beaker, was with standardized 0.1 N $BaCl_2$ soon after adding 1 or 2 small drops of *ca.* 0.001 per cent Na rhodizonate (Eastman) made up within ¾ of an hour before the titration. The end-point was a sharp bluish red. The accuracy was within 2 per cent as shown by duplicates and recoveries from standard solutions.

The *carbonic anhydrase activity* of the gills and various tissues was measured according to the method described by Meldrum and Roughton (1933), Brinkman, Margaria, and Roughton (1933), and Ferguson, Lewis, and Smith (1937). Extraction was performed by freezing the briefly water-rinsed tissues, macerating thoroughly while frozen, and then suspending in ten times their weight of distilled water. The extracts, with a drop of toluene, were kept in a refrigerator at about 5°C. for about 20 hours before use. The manometer bath was set at 15°.

DISCUSSION

One of the first questions which arises is whether the electrolytes are taken up as molecules or as ions. It is shown, in the present work, that the crayfish can take up the anion component of a dissolved electrolyte independently of the cation component and *vice versa*. Taking a specific example, Cl^- is absorbed from KCl and K^+ is left behind. One might, however, suppose that the Cl^- is absorbed as HCl and that the K^+ is left behind with the hydroxyl moiety of the water molecule. In support of such a supposition is the low electrical conductivity of certain cell walls and protoplasmic membranes, which, evidently, can permit but little dissociation (see the reviews of Osterhout, 1933, 1936). The following phenomena are against such a theory: (1) The bioelectrical effects imply appreciable permeability of the bounding membranes to certain ions at least (Osterhout, 1933[2]); (2) HCl and other mineral acids are known to enter living cells with difficulty, especially as compared with metallic hydrates (Jacobs, 1922; Osterhout, 1936; and others).

It is here shown that K^+ is not absorbed but that Na^+ is absorbed. It is thus clear that the uptake of ions is not necessarily correlated with their mobilities.

The uptake of Cl^- from KCl, from which the cation is not absorbed, and the uptake of Na^+ from Na_2SO_4, in which the anion is not absorbed, probably implies two separate mechanisms: one which takes up the cation and the other the anion. The fact that Cl^- is absorbed generally at a faster rate in NaCl than in KCl or $CaCl_2$, from which the metallic ions are either

[2] Osterhout, W. J. V., Permeability in large plant cells and in models, *Ergebn. Physiol.*, 1933, **35**, 1012, 1013.

not absorbed (Ca^{++}, ordinarily, and K^+) or else are absorbed independently of the Cl^- (Ca^{++} in newly molted and about-to-molt individuals), indicates that Na^+ and Cl^- are taken up largely together in molecular form—which may require a third type of mechanism. Since Ca^{++} is taken up only during specified periods, and then at a notably higher rate than that generally observed for Na^+ or Cl^- when such are absorbed alone, it appears that there is a fourth type of mechanism for Ca^{++} absorption. It is possible that through the agency of some periodically secreted hypodermal enzyme, Ca is deposited as $CaCO_3$ at the cuticle and might not actually enter the body. Reasons are given for the belief that the Na^+ and Cl^- are absorbed by the gills.

Our results support the statement of Krogh (1937 *c*) that "the selectivity of the transport mechanism is too high" to be readily explicable on the basis of an E.M.F. as the driving force, and Osterhout (1936) has pointed out that "if the surface charge determined the entrance of electrolytes it is difficult to see how both anions and cations could enter in equal numbers, as happens in *Valonia* [a marine alga]; for a negative charge would inhibit the entrance of anions and a positive charge that of cations." As already stated, we have presented good evidence that Na^+ and Cl^- are taken up together as NaCl or else are taken up in such a way that they mutually aid each other. This is apparently inexplicable by surface charges.

In reference to the marine alga, *Valonia*, Osterhout (1936 and earlier) has piled up considerable evidence which suggests that the accumulation of K^+ in this organism can be explained on the basis of the higher chemical potential of K^+, OH^- in the sea water than in the cell sap, the KOH activity gradient being due to acid metabolites of the cell sap which keep the pH of the sap markedly lower than that of the sea water. Such an explanation is admittedly inapplicable to fresh water forms (Jacques and Osterhout, 1935), in which no such differences in pH exist and in which the concentration gradients are much steeper (see also Steward, 1935, and Hoagland and Broyer, 1940).

The active transport of inorganic molecules is doubtless intimately correlated with the metabolism of the cell but no general explanation for such has as yet appeared. At present, the simplest explanation for the uptake of Cl^- from KCl (in which K^+ is left behind) is the assumption that this occurs in exchange for catabolic HCO_3^- and that the uptake of Na^+ from Na_2SO_4 (in which $SO_4^=$ is left behind) occurs in exchange for catabolic NH_4^+. (According to Brunow, 1911, 30–40 per cent of the total non-protein nitrogen eliminated by the crayfish is NH_3-N; according to Delaunay. 1931 and earlier, about 60 per cent of the non-protein nitrogen in crayfish

urine is NH_3-N.) No mere ionic exchange, however, seems capable of explaining why the uptake of Cl from NaCl (in which both Na^+ and Cl^- are taken up) is generally greater than the uptake of Cl^- from KCl (in which the cation is left behind). The absorption of Na^+ and Cl^- is so intimately correlated as to make it evident that both ions are absorbed largely as one molecule.

Krogh (1939) has remarked that the crayfish (*Potamobius astacus*) does not take up Ca^{++}; it appears very probable, from the present work, that he was dealing only with hard-cuticled animals not preparing to molt. The work of Irvine and Woodhead (1889) indicates that the shore-crab (presumably *Carcinus maenas*) requires $CaCl_2$ in sea water to harden its cuticle. Robertson (1937) has shown, by macro-analysis of samples of the natural sea water at intervals of about 7 days, that the soft-cuticled post-molt *C. maenas* absorbs notable quantities of Ca^{++} from the sea water. In addition, some Cl^- absorption was recorded. Since the diffusible, *i.e.* non-protein bound, blood-Ca^{++} of these animals is considerably higher than that of the sea water (Robertson) it is clear that even a marine crustacean can take up Ca^{++} from the exterior against the concentration gradient. The gradient, however, is relatively shallow in this case.

That the gastroliths can be of no significance in the calcification of the cuticle, due to the small quantity of calcareous material of which they are composed, has long been realized (Oesterlen, 1840; van der Hoeven, 1846–55). In *Cambarus clarkii* the raw ash which constitutes both of the apparently fully developed gastroliths is only about 3.16 per cent of the total ash in the integument of a hard-cuticled animal.

SUMMARY

1. Reasons are given for believing that the uptake of Na^+, Cl^-, and NaCl by the crayfish occurs through the gills.

2. A crayfish in fresh water, with a Cl concentration of about 0.2 mEq./l., can, by active Cl absorption, compensate entirely for Cl lost in the urine.

3. The carbonic anhydrase activity of the gills is markedly higher than that of other tissues of the crayfish, but the equivalent CO_2 output of the crayfish is far in excess of the equivalent Cl absorption per unit time and weight and thus fails to warrant the supposition that Cl absorption is of respiratory importance.

4. The carbonic anhydrase activity of the soft integument of the lobster, before and after molting, and of the hypodermis of the hard-cuticled animal is almost identical and of the same order as that of other tissues of the lobster.

5. The concentration of the electrolytes was about 7.5 mEq./l.; *i.e.*, considerably lower than in the blood of the crayfish. Cl^- can be taken up independently of the complementary cation. Na^+ can be taken up independently of the complementary anion. K^+ and $SO_4^=$ are not taken up at all. In pure NaCl, the Na^+ and Cl^- are absorbed evidently largely together. Ca^{++} is absorbed only in newly molted animals and in animals preparing to molt but is not absorbed by hard-cuticled animals not preparing to molt. Ca^{++} is taken up independently of Cl^- in pure $CaCl_2$.

6. Newly molted animals absorb Ca^{++} at a rate exceeding that of the absorption of other absorbable ions (Na^+ and Cl^-) in the same equivalent concentration.

7. A crayfish utilizes the Ca^{++} in fresh water in the calcification of its cuticle. Since the animal does not swallow water, the Ca^{++} must enter through the exterior. Reasons are given for believing that, unlike Na^+ and Cl^-, Ca^{++} is absorbed directly from the exterior by the integument and does not enter the body through the gills.

8. During molting, only about 4 per cent of the raw ash and 2.3 per cent of the organic material of the old cuticle is resorbed.

REFERENCES

Bogucki, M, Recherches sur la régulation de la composition minérale du sang chez l'écrevisse (*Astacus fluviatilis* L.), *Arch. internat. physiol.*, 1934, **38**, 172.

Brinkman, R., Margaria, R., and Roughton, F. J. W., The kinetics of the carbon dioxide-carbonic acid reaction, *Phil. Tr. Roy. Soc. London, Series A*, 1933, **232**, 65.

Brunow, H., Der Hungerstoffwechsel des Flusskrebses (*Astacus fluviatilis*), *Z. allg. Physiol.*, 1911, **12**, 215.

Cuénot, L., Études physiologiques sur les crustacés décapodes, *Arch. biol.*, 1895, **13**, 245.

Delaunay, H., L'excrétion azotée des invertébrés, *Biol. Rev.*, 1931, **6**, 265.

Ferguson, J. K. W., Lewis, L., and Smith, J., The distribution of carbonic anhydrase in certain marine invertebrates, *J. Cell. and Comp. Physiol.*, 1937, **10**, 395.

Griffiths, A. B., On the blood of the Invertebrata, *Proc. Roy. Soc. Edinburgh*, 1890–91, **18**, 288.

Hibbard, P. L., Hoagland, D. R., and Davis, A. R., The influence of light, temperature, and other conditions on the ability of *Nitella* cells to concentrate halogens in the cell sap, *J. Gen. Physiol.*, 1926, **10**, 121.

Hoagland, D. R., The absorption of ions by plants, *Soil Sc.*, 1923, **16**, 225.

Hoagland, D. R., and Broyer, T. C., Hydrogen-ion effects and the accumulation of salt by barley roots as influenced by metabolism, *Am. J. Bot.*, 1940, **27**, 173.

van der Hoeven, J., Handbook of zoology, Cambridge, The University Press, 1846–55, English translation by W. Clark in 1856.

Huf, E., Über den Einfluss der Narkose auf den Wasser- und Mineralhaushalt bei Süsswassertieren, *Arch. ges. Physiol.*, 1935, **235**, 129.

Irvine, R., and Woodhead, G. S., Secretion of carbonate of lime by animals. II, *Proc. Roy. Soc. Edinburgh*, 1889, **16**, 324.

Jacobs, M. H., The influence of ammonium salts on cell reaction, *J. Gen. Physiol.*, 1922, **5**, 181.

Jacques, A. G., and Osterhout, W. J. V., The kinetics of penetration. XI. Entrance of potassium into *Nitella*, *J. Gen. Physiol.*, 1935, **18**, 967.

Koch, H., Essai d'interprétation de la soi-disant "réduction vitale" de sels d'Argent par certains organes d'Arthropodes, *Ann. Soc. sc. Bruxelles, Series B*, 1934, **54**, 346.

Koch, H., The absorption of chloride ions by the anal papillae of Diptera larvae, *J. Exp. Biol.*, 1938, **15**, 152.

Koch, H., and Krogh, A., La fonction des papilles anales des larves de diptères, *Ann. Soc. sc. Bruxelles, Series B*, 1936, **56**, 459.

Krogh, A., Osmotic regulation in the frog (*R. esculenta*) by active absorption of chloride ions, *Skand. Arch. Physiol.*, 1937*a*, **76**, 60.

Krogh, A., Osmotic regulation in fresh water fishes by active absorption of chloride ions, *Z. vergleich. Physiol.*, 1937*b*, **24**, 656.

Krogh, A., Animal membranes, *Tr. Faraday Soc.*, 1937*c*, **33**, 912.

Krogh, A., The active absorption of ions in some freshwater animals, *Z. vergleich. Physiol.*, 1938, **25**, 335.

Krogh, A., Osmotic regulation in aquatic animals, Cambridge, The University Press, 1939, 242 pp.

Lienemann, L. J., The green glands as a mechanism for osmotic and ionic regulation in the crayfish (*Cambarus clarkii* Girard), *J. Cell. and Comp. Physiol.*, 1938, **11**, 149.

Linderstrøm-Lang, K., Studies on enzyme histochemistry. XIX. Microestimation of alkalies in tissue, *Compt.-rend. trav. Lab. Carlsberg, série chimique*, 1936, **21**, 111.

Lundegårdh, H., Untersuchungen über die Salzaufnahme der Pflanzen. III. Quantitative Beziehungen zwischen Atmung und Anionaufnahme, *Biochem. Z.*, Berlin, 1933, **261**, 235.

Lundegårdh, H., and Burström, H., Untersuchungen über die Anionenatmung, *Biochem. Z.*, Berlin, 1937, **290**, 104.

Maluf, N. S. R., The permeability of the integument of the crayfish (*Cambarus bartoni*) to water and electrolytes, *Biol. Zentr.*, 1937*a*, **57**, 282.

Maluf, N. S. R., Studies on the respiration (and osmoregulation) of animals. I. Aquatic animals without an oxygen transporter in their internal medium, *Z. vergleich. Physiol.*, 1937*b*, **25**, 1.

Maluf, N. S. R., On the anatomy of the kidney of the crayfish and on the absorption of chlorid from freshwater by this animal, *Zool. Jahrb., Abt. allg. Zool. u. Physiol. Tiere*, 1939, **59**, 515.

Meldrum, N. U., and Roughton, F. J. W., Carbonic anhydrase. Its preparation and properties, *J. Physiol.*, 1933, **80**, 113.

Nagel, H., Die Aufgaben der Exkretionsorgane und der Kiemen bei der Osmoregulation von *Carcinus maenas*, *Z. vergleich. Physiol.*, 1934, **21**, 468.

Oesterlen, F., Ueber den Magen des Flusskrebses, *Arch. Anat., Physiol. u. wissensch. Med.*, 1840, 387.

Øllgaard, E., Eine mikrotitrimetrische Methode zur Bestimmung von Sulfaten im Plasma, *Biochem. Z.*, Berlin, 1934, **274**, 181.

Osterhout, W. J. V., Permeability in large plant cells and in models, *Ergebn. Physiol.*, 1933, **35**, 967.

Osterhout, W. J. V., The absorption of electrolytes in large plant cells, *Bot. Rev.*, 1936, **2**, 283.

Robertson, J. D., Some features of the calcium metabolism of the shore crab (*Carcinus maenas* Pennant), *Proc. Roy. Soc. London, Series B*, 1937, **124**, 162.

Rosenfels, R. S., The absorption and accumulation of potassium bromide by *Elodea* as related to respiration, *Protoplasma*, 1935, **23**, 503.

Schumann, F., Experimentelle Untersuchungen über die Bedeutung einiger Salze, insbesondere des kohlensauren Kalkes, für Gammariden und ihren Einflusz auf deren Häutungsphysiologie und Lebensmöglichkeit, *Zool. Jahrb., Abt. allg. Zool. u. Physiol. Tiere*, 1928, **44**, 623.

Siwe, S. A., Einige Methoden zur Bestimmung von Ca in kleinen Blutmengen, *Biochem. Z.*, Berlin, 1935, **278**, 442.

Steward, F. C., The absorption and accumulation of solutes by living plant cells. V. Observations upon the effects of time, oxygen and salt concentration upon absorption and respiration by storage tissue, *Protoplasma*, 1933, **18**, 208.

Steward, F. C., Mineral nutrition of plants, in Luck, J. M., Annual review of biochemistry, Annual Reviews, Inc., Stanford University, 1935, **4**, 519.

Wang, C. C., Improvements in the methods for calcium determination in biological material, *J. Biol. Chem.*, 1935, **111**, 443.

KINETICS OF THROMBIN INACTIVATION AS INFLUENCED BY PHYSICAL CONDITIONS, TRYPSIN, AND SERUM*

By ANTHONY J. GLAZKO and JOHN H. FERGUSON

(*From the Laboratories of Pharmacology, University of Michigan, Ann Arbor*)

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It has been known since the time of Alexander Schmidt (32) that thrombin disappears rapidly from the serum after blood clotting. Isolated prothrombin and thrombin preparations vary in stability on keeping and on heating (8, 21, 30), and thrombinolysis by added trypsin is known to reduce its effectiveness in clotting blood (7, 11). A study of the kinetics of the reduction in coagulant activity of thrombin preparations, (*a*) held under controlled physical conditions, (*b*) as influenced by added trypsin, and (*c*) in the presence of serum, might be expected to shed light on the mechanism of thrombin inactivation.

Any factor which prolongs the clotting time of fibrinogen by thrombin might very well be termed an "antithrombin," but the term suffers from various ill-defined restrictions of usage. A distinction may be drawn between thrombin-inhibitors which produce their full effect *immediately* and those which act *progressively*. Examples of "immediate" antithrombic action are the effects of pH, salts, etc., especially including polyvalent anions and heparin (12, 16). Thrombinolysis is essentially a "progressive" phenomenon, and since the progressive character of the classical antithrombins has long been recognized (4, 17), there is obvious need for clarification of the relations between thrombinolysis and the antithrombic action of serum *in vitro* and *in vivo*. The present enquiry into the general characteristics of the process of progressive thrombin inactivation (*in vitro*) may assist in the approach to these problems.

I

Reagents and Methods

Full details of our routine methods of preparing the various clotting agents are given in a previous publication (9). Appended are some addi-

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tional technical considerations relating to modifications of the methods and the special requirements of the current investigations:

Preservation of Reagents; Apparatus Used; etc.—Low temperatures (ice box) are used for the preservation of solutions. In all cases, a few crystals of thymol are added to prevent bacterial growth. The glass electrode (Beckman pH meter) is used to control pH, and the specified temperatures are maintained (±0.5°C.) with a thermostat water bath. A mechanical rocking device and stop watch are used in timing coagulation (see below).

Prothrombin Solutions.—The Howell-type "prothrombin papers," each corresponding to 5 cc. of filtrate from defibrinated (55°C.), Berkefelded, citrated dog plasma, are extracted in the cold for 1–2 hours with distilled water and a drop or two of 0.5 per cent $NaHCO_3$. The pH of the filtered extract is adjusted to pH = 7.2 with N/10 acetic acid.

Activity.—In some recent tests, kindly performed for us by Dr. H. P. Smith (Iowa), it was found that the activity of such prothrombin solutions represents some 57 per cent of a corresponding volume of (average) dog plasma. 1 cc. of solution (3 papers + 60 cc. water) contained 1.25 mg. (total) N and assayed 47 prothrombin "units" (Smith *et al.* (36)); *i.e.*, activity = 38 units per mg. N.

The nitrogen (protein) content of the prothrombin solution was sometimes reduced by submitting the above salt-poor extract to isoelectric precipitation (pH = 5.3). On redissolving to original volume and routine pH (7.2), there was no significant loss in potency. Other prothrombin methods were tried, *e.g.* Mellanby's (21); also various adsorbed and eluted preparations (*e.g.* (34)), without finding any significant modification of the properties to be described. There was a suggestion that preliminary Berkefeld filtration of the plasma favored the subsequent preparation of a more stable prothrombin, but details have not been worked out.

Thrombin.—Is formed from prothrombin solution by activating to maximal coagulant activity with optimal amounts of N/10 $CaCl_2$ and a thromboplastic agent (cephalin or brain thromboplastin—see below). The pH is finally adjusted to 7.0 (unless otherwise stated). Like Mellanby (21) and others (35), we found that the final thrombin could be precipitated by acetone. The precipitate, whether dried immediately or kept under the acetone, has yielded active preparations, even after several months (ice box).

Thromboplastin.—The cephalin has been described previously (9). After some 2 years under alcohol it suffered considerable loss of potency and yielded solutions of noticeable acidity. A crude but potent thromboplastin is prepared by grinding up dog brain (fresh or frozen) with distilled water, diluting, and centrifugalizing. A desiccated preparation was also made by precipitating a saline (0.85 per cent NaCl) extract of brain with four volumes of acetone, recovering the deposit on a Büchner funnel, and drying at room temperature. Aqueous suspensions were made up as required.

Fibrinogen.—$(NH_4)_2SO_4$-fibrinogen is made from Berkefelded plasma in the usual way (9). A low salt content favors a sharp end-point in the clotting test (see below); hence the use of water, rather than saline, as the solvent and the avoidance of citrate (or oxalate), which might be desirable, theoretically, to control traces of prothrombin (8). Actual tests reveal little or no prothrombin, provided that the fibrinogen is made from Berkefelded plasma several days old and particularly if a well diluted fibrinogen solution is employed. It is unnecessary to resort to the previously recommended adsorption techniques (9) for getting rid of the last traces of prothrombin. Lyophilized

fibrinogen (10) has been found to lose all traces of prothrombin on simple keeping, but was not used in the current experiments. A new and easy method of preserving fibrinogen consists in dissolving the final salted-out protein in 20 per cent urea solution. Stored in the ice box, such preparations keep for several weeks. At low temperatures the urea protects the fibrinogen from denaturation, according to Diebold and Jühling (5). It is restored to a state closely resembling the original on removing the urea (*a*) by dialysis through Visking casing or (*b*) merely by diluting (10–30 times) to a point at which the urea ceases to exert a significant influence on clotting (16). Traces of prothrombin, if present in the fibrinogen, are apt to be preserved also and occasionally lead to some thrombin formation which causes traces of clot to appear in the recovered fibrinogen. Any unstable fibrinogen preparation, of course, is discarded.

Crystalline Trypsin.—Is now available commercially.[1] The preparation we used contained 0.022 mg. protein N per mg. of sample.

Serum.—The mode of preparation and testing of serum antithrombin will be described in the appropriate section.

Barbiturate Buffer.—In certain experiments, involving alteration of the pH of the thrombic mixtures, constancy of the hydrogen ion concentration in the actual thrombin-fibrinogen interaction was secured by adding an equal volume of N/10 barbital-Na (Michaelis (24)), at the routine pH (7.2), to the fibrinogen solution, and checking with the glass electrode. We have also used the expensive *imidazole buffer* recommended by Mertz, Seegers, and Smith (23), in several of the cited experiments.

Clotting Time and Thrombin Concentration (Relative): the "Inverse Law"

All methods of thrombin (and prothrombin) determination are necessarily relative, since the agent has not yet been defined chemically. In the current investigation, thrombin "concentrations" are initially expressed in terms of the degree of dilution of the *same preparation*, used as the standard of reference throughout the particular experiment. We have avoided any attempt to transfer the quantitative data from one experiment to another by means of arbitrary thrombin units (*cf.* 6).

The index of activity of thrombin in a given test is the clotting time (see below) for a suitable fibrinogen solution. The dependence of clotting time upon physical conditions (temperature, pH, salt content, fibrinogen concentration, etc.) makes it imperative to control all these variables throughout each series of tests. The influence of naturally-occurring antithrombins is also an important variable which will receive special consideration in other sections of this paper.

Many previous workers (*e.g.* 14) have established the general principle known as the inverse law, which states that the clotting time is an inverse (reciprocal) function of thrombin concentration. This expression is analogous to the well known Arrhenius "*Qt* rule" (1, 2), developed for re-

[1] Plaut Research Laboratory, Lehn and Fink Products Corporation, Bloomfield, New Jersey.

lating enzyme concentration to the time required for the production of a given amount of effect. The formulation reads

$$E \cdot y = K \quad (1),$$

i.e. the product of enzyme concentration E by time y is a constant (K). Alternatively,

$$E = \frac{K}{y} \quad (2),$$

i.e. enzyme concentration bears an inverse relation to the time factor. Without committing oneself to any hypothesis of the actual identification

TABLE I

Relation of Clotting Time to Thrombin Concentration

	E (relative concentration)	y	$E \cdot y$	$E\,(y - a)$
		sec.		
A				Where $a = -5$
	100	20	2000	2500
	80	25	2000	2400
	60	35	2100	2400
	40	55	2200	2400
	20	115	2300	2400
B				Where $a = 18$
	64	25	1600	448
	32	29	928	353
	16	45	720	430
	8	72	576	430
	4	105	420	348

A = Mellanby-type (21) thrombin.
B = Howell-type prothrombin (9) + Ca + brain thromboplastin.

of thrombin with the enzymes (*cf.* 27), "thrombin" may be substituted for "enzyme" in the mathematical analysis of the data.

When applying the formula to coagulation data, we may question the validity of postulating the "given amount of effect," *viz.* here the identical extent of change in fibrinogen at the moment of coagulation, irrespective of the amount or type of thrombin used. Actually, the only evidence for this is the reasonably close adherence to the inverse law throughout the range of thrombin concentrations with which we are working. There are undoubted empiricisms of technique, particularly in the method of timing the clotting.

We time the appearance of the first visible strands of fibrin in a relatively weak and salt-poor fibrinogen solution. This timing from the commencement of clotting is readily applicable to the translucent solutions with which we are working. It is particularly valuable for the weakest thrombins, which may fail to cause gel formation. Methods of timing based on solidity (firmness) of the clot (*e.g.* time when tube can be inverted without spilling the contents), while frequently used for such turbid materials as whole blood, etc., are obviously subject to additional variables such as the adhesiveness and coherence of the fibrin gel and the diameter of the tube used in the clotting test.

To test the applicability of equation 2, serial dilutions of a typical thrombin preparation were used. 0.5 cc. of each dilution was added to 1.0 cc. of weak fibrinogen solution at the routine temperature (20°C.) and pH (7.2), and the clotting time noted. The results are listed in Table I, Series A.

The product of clotting time by concentration (relative dilution) of thrombin gives a value which is constant within the limits imposed by the experimental technique. Equation 2 does fit the experimental data and, therefore, affords a means of assaying thrombic activity by timing the clot. Thrombin concentrations are henceforth computed from the actual clotting time data, the validity being checked by a similar series of dilution experiments accompanying each of the subsequent investigations.[2]

[2] Equation 2 represents a special case of the general equation for a linear relation,

$$E = \left(\frac{1}{y} - b\right) + m \qquad \text{(i)},$$

where b is a constant determined by the Y-axis intercept of the line obtained by plotting $\frac{1}{y}$ against E, and m is the slope of the line. $b = 0$ in the special case of equation 2, and, since b is usually quite small, this equation affords a sufficiently good approximation to the actual experimental conditions to serve the purposes of the present paper. In some of the data, closer agreement with the theoretical conditions is attained by the use of the general formula. Thus, the derived expression for the rate constant (k, equation 6) would be modified to

$$k = \frac{2.3}{(t_2 - t_1)} \log_{10} \frac{\left(\frac{1}{y_1} - b\right)}{\left(\frac{1}{y_2} - b\right)} \qquad \text{(ii)}.$$

A more manageable form of equation (ii) may be developed from the observation that K ($= Ey$; equation 1) varies directly with E, whence

$$K = Ey = aE + c \qquad \text{(iii)},$$

or

$$E = \frac{c}{y - a} \qquad \text{(iv)},$$

where c is a constant, determined by the intercept with the Y-axis of the line obtained on plotting K against E, and a is the slope. The *modified rate equation* now becomes

Kinetics of Thrombin Inactivation: Order of the Reaction

Provided we know the order of the reaction, the rate of the progressive inactivation of thrombin can be defined by the constant of the rate equation. The expression for a first order inactivation process is

$$-\frac{dE}{dt} = k \cdot E \tag{3}$$

or, integrating between the limits of the incubation times t_1 and t_2 (and corresponding thrombin concentrations E_1 and E_2)

$$-2.3 \log_{10}\left(\frac{E_2}{E_1}\right) = k\,(t_2 - t_1) \tag{4}$$

Substituting $\frac{K}{y}$ for E (equation 2), we obtain

$$-2.3 \log_{10}\frac{y_1}{y_2} = k\,(t_2 - t_1) \tag{5}$$

or

$$k = \frac{2.3}{(t_2 - t_1)} \log_{10}\left(\frac{y_2}{y_1}\right) \tag{6}$$

where k is the velocity constant for a first order reaction, representing the fraction of thrombin inactivated in unit time (a minute being used as the time unit throughout this paper).

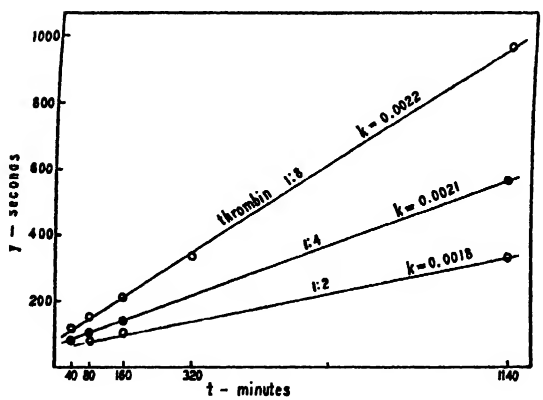

FIG. 1. Effect of thrombin concentration on the rate of inactivation (pH 7.0; temperature 38°).

The experimental results of Fig. 1 were obtained in a study of the rate of "spontaneous" inactivation of a thrombin prepared from the Howell-type prothrombin with $CaCl_2$ and brain thromboplastin, as described under reagents. The clotting time data were substituted into equation 6.

$$k = \frac{2.3}{(t_2 - t_1)} \log_{10}\left(\frac{y_2 - a}{y_1 - a}\right) \tag{v}$$

That this relation holds more widely than $E = \frac{K}{y}$, is shown by a comparison of the last two columns of Table I, especially for Series B. This expression may be used to extend the experimental validity of equation 6.

The values of y are routinely determined from the "best" straight line drawn through the points, using the method of least squares (22). The calculated k values are given in Fig. 1.

The values of k for different concentrations of thrombin are constant, within experimental limits. This has been confirmed repeatedly during these studies. It establishes the fact that the inactivation of thrombin follows the course of a first order reaction. Calculations based upon the assumption of zero order and of second order reactions gave variable values for k.

A generalized formulation of the results illustrated in Fig. 1 is

$$\frac{dy}{dt} = \frac{K'}{E} \tag{7}$$

This brings out the additional point that, although the fraction of thrombin inactivated in unit time is constant irrespective of the thrombin concentration, the *rate of increase in the clotting time* is greater for weak thrombin preparations than for stronger ones.

TABLE II

Variation of k for Different Thrombin Preparations

The thrombin preparations were made from the Howell-type prothrombin by activation with $CaCl_2$ and the designated thromboplastin. The values for k were obtained at pH 6.0 and 38°.

	Brain extract							Cephalin	
$k \times 10^4 =$	50*	20*	80*	10*	1	4	35	13*	60

* Acetone-precipitated thrombin (see reagents).

Variation of k for Different Thrombin Preparations: Influence of Thromboplastin

Although many types of active thrombin (crude) have been described in the literature (see section on methods), all preparations are empirical and make no claim to chemical purity. The data summarized in Table II show fairly wide variations of the k value for different thrombin preparations. This is true for a large number and variety of thrombin preparations which have been investigated. It even holds for solutions made on different occasions with exactly similar techniques of preparation. The question of the possible influence of the type of thromboplastic agent used in activation is especially important in view of the possibility that this may contribute a thrombinolytic factor (in crude tissue extracts, for instance).

Fig. 2 gives the complete activation, and subsequent inactivation curves for a *weak* prothrombin solution, converted into thrombin by a fixed amount of N/10 $CaCl_2$ and varying amounts of a cephalin preparation. There is some inhibitory effect on thrombin formation with the higher cephalin concentrations, due perhaps to the large excess of cephalin in relation to the weak prothrombin used (8). The chief effect appears to be immediate, since an analysis of the linear portion of the inactivation curves shows only a threefold increase in the k value for a 16-fold increase in cephalin concentration.

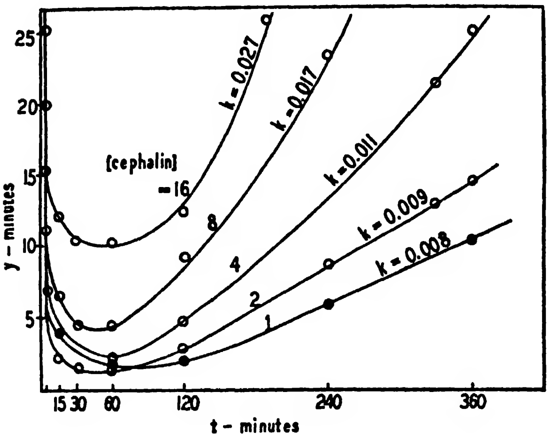

FIG. 2. Changes in coagulant activity produced by the activation of a weak prothrombin preparation with Ca^{++} and different concentrations of cephalin (pH 8.0; temperature 38°).

Table III shows the effects of varying amounts of (A) cephalin, added to fully formed thrombin and (B) brain thromboplastin, added to recalcified

TABLE III

Influence of Thromboplastin on k (pH = 7.5; temperature = 38°)

Clotting times (seconds) given in body of tables.

A. Cephalin Added to a Fully Formed Thrombin

Final concentration of cephalin	Incubation time (minutes) 30′	60′	120′	360′	k (approximate)
1:6000	44″	50″	65″	130″	0.003
1:12,000	42″	42″	53″	125″	0.003
1:24,000	42″	47″	62″	120″	0.003
None added	33″	37″	44″	130″	0.004

B. Brain Thromboplastin Added to a Weak Prothrombin (Recalcified)

Relative concentration of brain extract	Incubation time (minutes) after recalcification 6′	20′	60′	120′	280′	1440′	k (approximate)
9	41″	50″	70″	60″	68″	180″	0.0010
3	30″	43″	65″	65″	78″	250″	0.0015
1	28″	42″	70″	70″	100″	315″	0.0017

prothrombin. The k values for the respective inactivation processes again show very little dependence on concentration of the thromboplastic factor.

The addition of saliva (another thromboplastic agent (15)) had no effect on the velocity constant.

These studies are by no means exhaustive and should be extended to a wider variety of thromboplastic agents. For present purposes, the cited data suffice to uphold the contention that the major factor in the k variations of the individual thrombins is *not* significantly related to the thromboplastin.

II

Influence of Physical Conditions, Trypsin, and Serum.
Influence of Physical Conditions

(*A*). *Temperature.*—The older literature (27) contains frequent reference to the instability of thrombin at elevated temperatures. Table IV presents the results of kinetic studies on various thrombins at different temperatures.

The *critical thermal increment* (μ) was calculated from the integrated form of the van't Hoff-Arrhenius equation

$$\mu = \frac{4.6\ T_1T_2}{(T_2-T_1)} \log_{10}\left(\frac{k_2}{k_1}\right) \qquad (8),$$

where μ represents the critical thermal increment ("activation energy") in calories per mol, k_1 and k_2 are the velocity constants, calculated from equation 6, for the absolute temperatures T_1 and T_2, respectively.

The values for Q_{10} were computed from the formula

$$\log Q_{10} = \frac{10}{(T_2-T_1)} \log\left(\frac{k_2}{k_1}\right) \qquad (9).$$

The following points are brought out in the data:

1. Below 15°C., the rate of inactivation becomes very small and the increase of k with rise of temperature (as indicated by the Q_{10}) is very minor. This resembles many enzyme reactions (38). It also emphasizes the necessity of running clotting tests at low temperatures to prevent loss of thrombic activity (especially during the activation of weak prothrombins) during any prolonged clotting tests (9).

2. Between 20°C. and 40°C., the value of μ is of the same order as that obtained in many known chemical and enzymatic reactions (*e.g.* 38).

3. Above 50°C. (especially near 60°C.), the value of μ increases mark-

edly to over 40,000 calories per mol (at constant pH). Such high values (under similar conditions) are characteristic of the thermal denaturation of proteins (3) and the heat inactivation of enzymes (38) and viruses (18). There is, therefore, a definite suggestion that, at about 60°C., thrombin is directly influenced by heating. This would be the case if thrombin were a protein, or so closely associated with proteins as to be affected by their thermal denaturation. Strong thrombin preparations may retain a fair

TABLE IV

Influence of Temperature on k

Thromboplastin	Treatment	pH	T°C.	$k \times 10^4$	μ	Q_{10}
Cephalin	Acetone-thrombin	5.6	40.0	8		
			40.0	11	24,400	3.4
			50.0	37		
			50.0	39	22,500	2.8
			55.0	64		
			55.0	59		
			55.0	70		
Cephalin	Thrombin extracted with alcohol-ether	7.0	15.0	9	9,070	1.7
			38.0	30		
Cephalin	—	7.0	7.5	0		
			15.0	2	6,830	1.5
			25.0	3	18,500	2.8
			38.0	11		
Cephalin	—	7.2	49.0	32	39,800	7.8
			59.0	249		
Brain	Acetone-thrombin	7.2	7.5	0		
			20.5	5	10,700	1.8
			38.0	14		
Brain	—	7.2	49.0	9	71,000	27.7
			59.0	249		

degree of activity, however, even after boiling for a few minutes (8). The presence of water is apparently essential for denaturation (3). When dried, thrombin preparations are much more stable to heating (21) and storing (35, 30) than when in solution.

There is an unexplained variability between the different thrombin preparations (Table IV). In enzyme systems, which show analogous behavior, such variability is often associated with the impurities present in the preparations, although many of the essential data are the same for crude preparations as for highly purified enzymes (37). In analogous systems, we also have the suggestion of specific intramolecular groups which act as

negative catalysts (inhibitors), their effectiveness being lessened by dilution (18). Such an explanation may account for the greater stability of highly concentrated thrombin solutions, and for the marked deviations from the inverse law which they exhibit.

The k values for the data on temperature effects were obtained at constant pH. It has been pointed out that, for certain thermal denaturation reactions (*e.g.* pepsin), where the rate varies with the hydrogen ion concentration, the practice of measuring the critical thermal increment at constant pH gives an unduly high value due to the inclusion of "the heat of dissociation of all acidic equilibria involved in preparing the initial molecules for the kinetic step of activation" (La Mer, 20). Since our preparations are admittedly crude, we do not propose to elaborate upon temperature and pH interrelationships, but merely to state that the calculations for the critical thermal increment are subject to a correction. The effect of pH on the velocity constant is dealt with in the following section.

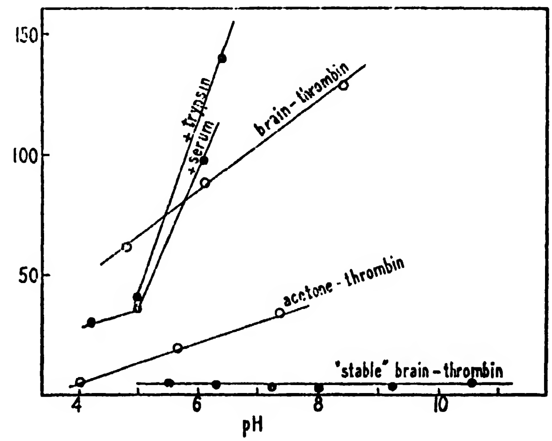

FIG. 3. Effect of pH on the velocity constants of various thrombin preparations (temperature 38°).

(*B*). *pH*.—In studying the influence of hydrogen ion concentration on the rate of thrombin inactivation, the thrombin preparations are adjusted to definite pH values (with the glass electrode) and the inactivation rate is measured at 38°C. All clotting tests are made on fibrinogen, buffered with barbital at pH = 7.2. The results are given in Fig. 3.

In all cases, the rate of inactivation below a pH of about 3.5 is too great for measurement. The acid-inactivated thrombin is not reactivated by neutralization. The most *stable* thrombin preparations show very little change of k between pH 5.0 to 10.0. *Unstable* preparations, including those to which trypsin or serum was added (see below), are most stable at pH of 4 to 5 and become less and less stable as the alkalinity is increased. The probable significance of these results will be brought out later, in the light of the additional experiments with trypsin and serum.

Influence of Added Trypsin

Crystalline trypsin (26) is a purified preparation of a well known protease, which has a recognized ability to destroy thrombin (7, 11).

In the experiments illustrated in Fig. 4, known amounts of crystalline trypsin were added to thrombin, at pH = 8.2 and temperature = 38°C. The k values were computed, as usual, from the clotting times, at various periods after the addition of trypsin. It is evident that the k values increase in direct proportion to the amount of trypsin added.

The similarity of the calculated and observed values for k in Table V is striking evidence in support of the postulate that the rate of thrombin inactivation is indeed directly proportional to the trypsin present, as well as to the concentration of thrombin (equation 3). The full results may now be expressed as follows:

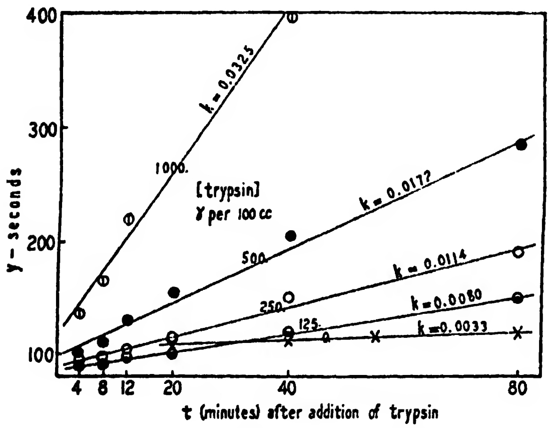

FIG. 4. Effect of trypsin on the velocity constant (pH 8.2; temperature 38°).

$$-\frac{dE}{dt} = k' \cdot E \cdot L \qquad (10),$$

differing from equation 3, in the introduction of the factor L, which represents the concentration of the thrombinolytic factor (trypsin). This equation describes a first order inactivation reaction and is a generalized equation representing the kinetics of progressive thrombin inactivation. It is obvious that once a thrombin unit is chosen, the lysin unit is readily defined.

Influence of Serum

The classical studies of Howell (17), and the long-forgotten but significant data of Collingwood and MacMahon (4), include mention of the progressive character of the antithrombic action of serum. Heating serum to 60°C. for a few minutes is said to destroy most of the antithrombic properties. Howell's contention that the action is associated with the serum proteins is supported by Quick (29), who, contrary to Howell and his pupils, finds a thermolabile "albumin" fraction effective. In our earlier experiments (12) no significant antithrombic action was demonstrable with a crude plasma albumin (*sans* heparin). We have recently (13) confirmed this by

incubating similar crude plasma albumin with a stable thrombin for a full hour, at 24°C., and noting only a 15 second increase over the original clotting time of 18 seconds (maintained unchanged in the control). Tests have also been made with a pure crystalline serum albumin, kindly supplied by Dr. T. L. McMeekin (Harvard), which fail to demonstrate any significant antithrombic action alone, *and* (in sharp contrast to crude albumin) in the presence of heparin. We subscribe, therefore, to the belief that antithrombin is not serum albumin, but some unknown factor which may or may not be associated with it.

Since Quick's experiments (29), like those of Morawitz (25), support claims for antithrombin in plasma by procedures involving a preliminary

TABLE V

The Relationship between k and the Concentration of Trypsin

(Data Taken from Fig. 4)

Trypsin micrograms per 100 cc.	$k \times 10^4$ observed	$k \times 10^4$ calculated*
1000	325	314
500	172	174
250	114	104
125	80	69
0	33	34

* The *calculated* value for k was obtained by assuming that a direct relationship existed between k and the concentration of trypsin. The equation for the straight line was used:

$$y = ax + b$$

where $y = k$; $a = 0.028$; $b = 3.4$;
and $x =$ the trypsin concentration.

defibrination, it cannot be asserted with any degree of certainty that untreated plasma contains an active antithrombin. Some of our results indicate that the antithrombin in plasma is present as an inactive substance.

The present kinetic studies on serum antithrombin involve two methods, (*a*) following the concentration of thrombin present in serum from the earliest practicable moment after defibrination, and (*b*) adding varying amounts of a thrombin-free (tested) serum to a known thrombin preparation.

Preparation of Serum.—Acetone-thromboplastin (see reagents) is added to citrated plasma diluted with 0.85 per cent NaCl, and recalcified with an optimal amount of $CaCl_2$. The fibrin is removed with a glass rod as fast as it is formed. The addition of a few drops of thrombin to a sample of the final serum confirms the completeness of the defibrination.

Tests.—(*a*) Immediately upon completion of the defibrination, 0.5 cc. of serum is added to 1 cc. of fibrinogen solution, under the conditions previously described for thrombin assay, and the test is repeated at various time intervals. (*b*) For the second series of experiments, increasing amounts of serum (thrombin-free, by tests on fibrinogen) are added to thrombin and the kinetics followed in the usual way.

(*a*).—Table VI shows that the inactivation rate constant (k) for the naturally occurring thrombin of serum is exceedingly high. The inactivation rate for thrombin in serum appears to be directly dependent upon the initial concentration of the plasma.

Hemophilia.—The serum from a citrated hemophilic plasma was diluted tenfold and clotted by $CaCl_2$, both with and without added thromboplastin.

TABLE VI

Rate of Thrombin Inactivation in Serum (pH = 7.4; Temperature = 38°)

Brain thromboplastin was added to dog plasma before recalcifying. Incubation time is given in minutes, measured from the time of recalcification. The clotting time is given in seconds in the body of the table.

Dilutions of plasma	Incubation time									$k \times 10^4$
	1.5′	2′	2.5′	3′	4′	5′	6′	6.5′	8′	
1:1	—	—	—	2″	27″	—	∞	—	—	Very high
1:2	—	19″	23″	33″	77″	102″	135″	—	—	4690
1:4	22″	30″	—	45″	68″	93″	113″	—	—	3060
1:8	—	22″	—	33″	50″	73″	—	105″	135″	2800

In each case the k value was 0.206. This indicates that the antithrombic factor is normal in hemophilic serum.

(*b*).—In Table VII, increasing amounts of thrombin-free serum were added to a thrombin solution and the kinetics of inactivation studied. The results clearly show the ability of serum to increase the thrombin inactivation rate. The k value rises in direct proportion to the amount of serum added. It may also be noted that the 24 hour old serum preparation used for the data of Table VII, Series B, is much weaker than the fresh serum of Table VII, Series A. This suggests a lability of the thrombinolytic factor in serum and lessens the bare possibility of the inactivation process being some sort of combination or adsorption on to the serum proteins (19, 31). The inability to reactivate serum-lysed thrombin with alkali or acid (*cf.* "metathrombin," 25, 17) also argues strongly against a non-lytic inactivation mechanism.

The ***rate of decrease of the antithrombic activity of serum*** was studied at different pH levels, but constant temperature (38°C.). 1 cc. of each serum

sample, held at the chosen pH for the cited times, was added to 5 cc. of a stable thrombin, buffered at pH = 7.6 with imidazole buffer (see reagents).

TABLE VII

Influence of Serum on k. pH = 7.2, Temperature = 38°.

Clotting times (seconds) given in body of tables.

A. Fresh Serum (Thrombin-Free)

Cc. serum added to 40 cc. thrombin	Saline	Incubation time (minutes)				$k \times 10^4$ (approximate)
		1′	3′	6′	11′	
	cc.					
2	14	68″	85″	112″	173″	1010
4	12	73″	103″	106″	∞	1560
8	8	90″	122″	∞	∞	1920
16	0	100″	160″	∞	∞	2350

B. Aged Serum (24 Hrs. at 9°C. Thrombin-Free)

Cc. of serum added to 40 cc. thrombin	Saline	Incubation time (minutes)							$k \times 10^4$ (approximate)
		0′	10′	21′	24′	39′	50′	66′	
	cc.								
0	2.00	40″	44″	—	66″	82″	103″	—	220
0.12	1.88	—	52″	68″	—	100″	—	170″	240
0.25	1.75	—	55″	72″	—	110″	—	210″	240
0.50	1.50	—	52″	78″	—	145″	—	∞	350
1.00	1.00	—	64″	95″	—	184″	—	∞	360
2.00	0	—	66″	135″	—	∞	—	—	650

TABLE VIII

Inactivation of Serum "Antithrombin"

The body of the table gives the ($k \times 10^4$) values obtained by the addition of 1 cc. serum (after the cited time of incubation, at 38° and the given pH) to 5 cc. of thrombin. The thrombin and fibrinogen solution were buffered with N/10 imidazole to pH 7.6.

pH	Incubation of serum (minutes)		
	30′	83′	137′
2.0	0	0	0
3.9	242	87	0
5.4	426	487	386
7.4	288	365	253
9.8	920	642	437

The thrombin inactivation rates were determined in the usual manner. The results are presented in Table VIII.

Although more antithrombic activity was initially present at pH = 9.8 than in sera kept at low pH values, there was more rapid loss of antithrombic activity at the most alkaline pH. At pH 5.4 and 7.4 a small increase in antithrombic activity is apparent. At pH values below 4.0, there was practically no antithrombic activity. This was not due to the previously described effect of acids on thrombin (see above), since the thrombin itself was well buffered at pH = 7.6. The sera kept for over 2 hours at pH levels of 2.0 and 3.9 did not regain their antithrombic activity during the incubation with the thrombin at pH = 7.6. However, the loss of antithrombin is reversible, under appropriate conditions, as shown by the data of Table IX.

Here the serum was brought to pH = 1.7. After standing for about 10 minutes, a portion was brought to pH = 9.6 and allowed to stand at

TABLE IX

The Reactivation of Serum Antithrombin

Clotting times (seconds) given in body of table.

pH of added serum	Incubation time (minutes) = serum + thrombin						$k \times 10^4$ (approximate)
	0.5′	3′	7′	11′	18′	24′	
1.7	88″	80″	78″	76″	73″	75″	—
Readjusted to 9.6	90″	92″	110″	145″	190″	250″	480
No serum added	90″	90″	88″	85″	80″	80″	—

38°C., for 30 minutes; the rest was kept (at pH = 1.7) as a control. 1 cc. of each sample was then added to 5 cc. of thrombin, at pH = 7.6, and the thrombin inactivation rate measured as before. The alkalinized preparation recovered a marked degree of antithrombic activity, whereas there was a complete absence of antithrombin in the acid sample, as seen by comparing with the thrombin control. The similarity of these pH effects to those obtaining with trypsin (Northrop, 26) is remarkable.

DISCUSSION

It is evident from the foregoing experiments that prepared thrombins undergo progressive inactivation, following the course of a first order reaction. By computing the velocity constant (k) from kinetic data, the process may be characterized independently of the factor of thrombin concentration. Since k may vary greatly in thrombins which are similar in initial activity, there is strong evidence for a thrombinolytic factor, which varies from preparation to preparation. The obvious suggestion is that it must be regarded as an "impurity" in the thrombin.

The proteolytic enzyme trypsin is an example of added impurity, which can alter the kinetic behavior of a *stable* thrombin to that of one which is much *less stable*. The so called antithrombic action of serum closely resembles that of trypsin in the studies we have made concerning its kinetic aspects. The physical conditions of temperature and pH, which are so important in thrombin formation, are no less significant in modifying thrombin inactivation. Whereas the most stable thrombins retain their activity over a wide pH range (5.0–10.0), the less stable preparations (including those to which trypsin or serum has been added) show a maximum stability at pH = 4–5.

The chief interest in the facts herein presented lies in the explanation which must be offered to account for them and in the future lines of enquiry to which they clearly point. Chief among the latter will be the attempt to isolate the thrombin impurity (antithrombin), which is indicated by the data. Pending the outcome of investigations now proceeding, by which it is hoped to characterize the thrombinolytic factor from the viewpoint of its proteolytic activities, we are advancing only tentatively the conclusion that progressive antithrombic action may be due to an enzyme impurity in the thrombin (and prothrombin) preparations.[3]

The following summarized points constitute the present evidence in support of this conclusion:

1. Added trypsin, like serum antithrombin, converts a stable thrombin into an unstable one, with a marked increase in the inactivation rate constant.
2. The acidic region (pH = 4–5) at which unstable thrombins are most slowly inactivated is the pH zone at which the effects of trypsin and serum are minimal. Northrop (26) has pointed out that pure trypsin is inactive in this pH region.
3. The inactivating agent (serum antithrombin) appears to be most stable at pH 5 to 6.
4. The unimolecular behavior of the inactivation process, together with the effects of varying physical conditions, are wholly consistent with the enzyme hypothesis.

It is not intended to place the whole burden of thrombin inactivation upon the postulated thrombinolytic enzyme. The slow deterioration of the

[3] A careful search of the literature, following the completion of this paper, brought to light very little to suggest an enzymatic nature of antithrombin, with the important exception of a long-forgotten contribution by Collingwood and MacMahon (4), to whom must be given due credit for conclusions very similar to those herein presented, even to the analogy between serum antithrombin and pancreatic trypsin.

more stable thrombins may very well be a protein denaturation. Thermal denaturation of proteins, enzymes, and viruses (some of which are now known to be proteins) follows a very similar course. The rapid inactivation, especially at moderate temperatures, in the physiological pH range, is the main reason, apart from the analogies outlined above, for postulating a specific thrombinolytic factor.

We do not know what thrombin is, but all the available evidence indicates that it is a protein, or so closely bound up with proteins as to be affected by their susceptibility to denaturation and proteolysis. Thrombin may also be an enzyme, although definite proof for, or against, this is still lacking (27). We have previously (11) advanced the idea that another type of enzyme, *viz.* a thromboplastic enzyme, accompanies (crude) prothrombin and, in conjunction with ionized calcium and phospholipid (cephalin), activates it to thrombin. The present data indicate yet another enzymatic function, namely, thrombinolytic. This need not complicate the picture unduly, if it should turn out to be identical with the thromboplastic factor, and especially if both are tryptic. Plasma tryptases are already known (Schmitz, 33) and might very well be expected to accompany plasma prothrombin and thrombin preparations. Schmitz's work raises the question of the rôle of specific inhibitors (antitrypsins), and kinase factors, in these new functions we are postulating for plasma tryptase. Upon the answer to this question may hinge the explanation of the continued fluidity of the blood *in vivo*.[4]

Tissue (including platelet and possibly leucocyte) sources of protease are not immediately involved in our Berkefelded plasma reagents, with cephalin as the only added thromboplastic agent. However, tissue antithrombins have been described in the literature. These may very well depend upon artificial methods of preparation and will require more extensive investigation than the data here presented. It may be significant that brain extracts are known to contain an antitrypsin (28). The data of Fig. 2 and Table III do not lend strong support to Howell's view that thromboplastin "neutralizes" antithrombin, but Howell himself had certain doubts, *e.g.*, the inability of cephalin to overcome the serum inhibitor of fully formed thrombin (17).

It is not proposed to take up space to contrast our thrombinolytic antithrombin theory with older views which postulate a loose thrombin-antithrombin "combination" (? metathrombin formation). Our inactivated thrombins are irreversible and unaffected by treatment with alkali

[4] Some of our recent experiments (Glazko, A. J., and Ferguson, J. H., *Proc. Soc. Exp. Biol. and Med.*, 1940, **45**, 43) show that heparin inhibits the action of trypsin on a casein substrate.

and acid (*cf.* metathrombin). All the evidence points to the progressive action of a specific antithrombin, rather than to the progressive production of an immediate antithrombin.

We have often observed that clots formed in the presence of unstable thrombins are especially liable to undergo fibrinolysis. Correlation of fibrinolysis with thrombinolysis is, therefore, suggested.

We are currently engaged in an attempt to use the basic methods of this paper in a simple clinical test for progressive antithrombins. Provided that a practical thrombin unit can be (empirically) established, a unit of antithrombin can readily be defined from equation 10.

SUMMARY

1. A new technique for studying the progressive inactivation of thrombin is described.

2. Thrombin inactivation follows the kinetics of a first order reaction.

3. The rate constant of the inactivation reaction increases with temperature and pH (5.0 → 10.0), and also with the presence of crystalline trypsin, or serum. The rate varies for different thrombin preparations, even under the same experimental conditions.

4. The temperature characteristics of the reaction indicate that thrombin is associated with protein.

5. Thrombin preparations are most stable at pH 4 to 5, even when trypsin or serum is added.

6. The progressive inactivation is believed to be due to two mechanisms: (1) a major effect, thought to be the action of a "serum-tryptase," which is usually present in the thrombin preparations, and (2) a minor effect, probably attributable to denaturation of thrombin-protein.

7. Sources of the thrombinolytic factor (serum-tryptase) and its implications in the general theory and practical problems of blood coagulation and antithrombic action are briefly discussed.

REFERENCES

1. Arrhenius, S., Quantitative laws in biological chemistry, London, G. Bell and Sons, Ltd., 1915, 45.
2. Astrup, T., *Enzymologia*, 1938, **5**, 119; also *Nature*, 1938, **141**, 1057.
3. Chick, H., and Martin, C. J., *J. Physiol.*, 1910, **40**, 404.
4. Collingwood, B. J., and MacMahon, M. T., *J. Physiol.*, 1913–14, **47**, 44.
5. Diebold, W., and Jühling, L., *Biochem. Z.*, Berlin, 1938, **296**, 389.
6. Eagle, H., *J. Gen. Physiol.*, 1935, **18**, 531, 547, 809, 813.
7. Eagle, H., and Harris, T. N., *J. Gen. Physiol.*, 1937, **20**, 543.
8. Ferguson, J. H., *Am. J. Physiol.*, 1937, **119**, 755; 1938, **123**, 341.
9. Ferguson, J. H., *J. Lab. and Clin. Med.*, 1938, **24**, 273.
10. Ferguson, J. H., and Erickson, B. N., *Proc. Soc. Exp. Biol. and Med.*, 1939, **40**, 425.

11. Ferguson, J. H., and Erickson, B. N., *Am. J. Physiol.*, 1939, **126**, 661.
12. Ferguson, J. H., *Proc. Soc. Exp. Biol. and Med.*, 1939, **42**, 33.
13. Ferguson, J. H., *Am. J. Physiol.*, 1940, **130**, 759.
14. Fischer, A., *Jap. J. Exp. Med.*, 1935, **13**, 223.
15. Glazko, A. J., and Greenberg, D. M., *Am. J. Physiol.*, 1938, **125**, 108.
16. Glazko, A. J., and Greenberg, D. M., *Am. J. Physiol.*, 1940, **128**, 399.
17. Howell, W. H., *Harvey Lectures*, 1916–17, **12**, 273.
18. Lauffer, M. A., and Price, N. C., *J. Biol. Chem.*, 1940, **133**, 1.
19. Lenggenhager, K., *Klin. Woch.*, 1936, **15**, 1835.
20. La Mer, V. K., *Science*, 1937, **86**, 614.
21. Mellanby, J., *Proc. Roy. Soc. London, Series B*, 1930, **107**, 271; 1933, **113**, 93.
22. Mellor, J. W., Higher mathematics for students of chemistry and physics, London and New York, Longmans, Green and Co., 1929.
23. Mertz, E. T., Seegers, W. H., and Smith, H. P., *Proc. Soc. Exp. Biol. and Med.*, 1939, **41**, 657.
24. Michaelis, L., *Biochem. Z.*, Berlin, 1931, **234**, 139.
25. Morawitz, P., *Beitr. chem. Physiol. u. Path.*, 1904, **4**, 381; also *Ergebn. Physiol.*, 1905, **4**, 307.
26. Northrop, J. H., Crystalline enzymes. The chemistry of pepsin, trypsin, and bacteriophage. Columbia Biological Series, No. 12, New York, Columbia University Press, 1939.
27. Oppenheimer, C., Die Fermente und ihre Wirkungen, Leipzig, 1926, **2**, 1140; *suppl.*, 1937, **7**, 951.
28. Page, I., Chemistry of the brain, Springfield, Illinois, Charles C. Thomas, 1937, 298.
29. Quick, A. J., *Am. J. Physiol.*, 1938, **123**, 712.
30. Roberts, A. C., *Proc. Soc. Exp. Biol. and Med.*, 1935, **32**, 606.
31. Scheuring, H., *Biochem. Z.*, Berlin, 1937, **291**, 1.
32. Schmidt, A., Zur Blutlehre, Leipzig, F. C. W. Vogel, 1892; Weitere Beiträge zur Blutlehre, Wiesbaden, J. F. Bergmann, 1895.
33. Schmitz, A., *Z. physiol. Chem.*, 1936, **244**, 89; 1937, **250**, 37; 1938, **255**, 234.
34. Seegers, W. H., Smith, H. P., Warner, E. D., and Brinkhous, K. M., *J. Biol. Chem.*, 1938, **123**, 751.
35. Seegers, W. H., Brinkhous, K. M., Smith, H. P., and Warner, E. D., *J. Biol. Chem.*, 1938, **126**, 91.
36. Smith, H. P., Warner, E. D., and Brinkhous, K. M., *J. Exp. Med.*, 1937, **66**, 801.
37. Sizer, I. W., *J. Biol. Chem.*, 1940, **132**, 209.
38. Stearn, A. E., *Ergebn. Enzymforsch.*, 1938, **7**, 1.

THE USE OF SOLUBILITY AS A CRITERION OF PURITY OF PROTEINS

I. Application of the Phase Rule to the Solubility of Proteins. II. The Solubility Curves and Purity of Chymotrypsinogen

By J. A. V. BUTLER*

(*From the Laboratories of The Rockefeller Institute for Medical Research, Princeton, New Jersey*)

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I. Application of the Phase Rule to the Solubility of Proteins

Since proteins cannot be heated and can only be treated with a limited range of solvents without injury, it has been inevitable that the methods of separation and characterization of different proteins should depend mainly on their behavior in aqueous solutions of varying acid and salt concentrations. In the early studies of Hardy (1) and Mellanby (2) it was found that the solubility of serum proteins varied with the amount of the saturating body, and Sörensen and his collaborators also found this kind of behavior with egg (3) and serum albumin (4) and casein (5). Sörensen seems to have been the first to apply the phase rule in the sense that for a single solute the solubility should be independent of the amount of the solid phase, and his inability to obtain fractions obeying this criterion led to his theory of "reversibly dissociating systems," according to which the real independent components of a protein were polypeptides which formed complex aggregations varying with the circumstances.

Since then a number of cases have been found in which the solubility is approximately independent of the amount of the solid phase (6), at least when there is a considerable excess of the latter. Northrop and Kunitz (7) have, however, shown that constant solubility in the presence of a large excess of solid is an insufficient criterion of purity, but if solubility curves (*i.e.*, solubility against total amount of saturating material) are determined in the region in which the excess of solid is small it may be possible to distinguish the solid phases present and in some cases to differentiate between mixtures of crystalline phases and solid solutions (8).

Few investigations of the purity of proteins have been made which satisfy

* Fellow of The Rockefeller Foundation.

these conditions and the object of the present work was to make a more exhaustive study of the solubility method as a criterion of purity of proteins.

Application of Phase Rule to Protein-Acid-Salt Solutions

In the first place it may be well to point out exactly what can be deduced from the phase rule. In the usual form this is written as $P + F = C + 2$, where P = number of phases, F = number of degrees of freedom, and C = number of components. We have first to choose as components the least number of substances by which we can express the composition of the solid protein phase or phases and the solutions which can be formed from them. Since the solutions with which we shall be concerned contain a buffering system, the least number of components will be four; *viz.*, protein, salt, acid, and water. Additional components may of course be introduced into the system at will, *e.g.* there may be two salts, such as the buffering salt and a neutral salt like ammonium sulfate. But it is unnecessary to regard a salt formed by the combination of the protein and the acid, even if the solid is present partly or completely as such, as a separate component, since its composition can be completely specified by the amounts of protein and acid contained in it. We can take as the component protein, the protein in a specified invariable condition and the amount of protein in any phase is determined by a suitable analysis; *e.g.*, of the amount of "protein nitrogen."

By the phase rule, this system of four components has five degrees of freedom in one phase, and four degrees of freedom when present in two phases. Four of these are the temperature, pressure, and concentrations of salt and acid respectively. When these four variable factors are fixed there remains one degree of freedom for a single phase and none for a system of two phases. In a homogeneous solution of a single protein the concentration of protein may vary, but if a solid phase is present the concentration of the dissolved protein is fixed. If it is not fixed then an additional component must be present. If it should happen that two distinct solid phases are present (*e.g.*, if a new phase were formed by reaction of the protein with the salt solution) the number of degrees of freedom would be further reduced by one; *i.e.*, it could only occur at a particular acid or salt concentration.

The characteristics of the possible systems which may be formed from one or two proteins together with water, acid, and salt are shown in Table I. When two proteins are present, a single solid phase may consist of either one protein, or a solid solution of the two proteins. In either case, since there are now five components in two phases, the number of degrees of freedom

under the stated conditions is one. Thus if the solid phase is one of the proteins, the concentration of the other in the solution is variable, while if the solid phase is a solid solution of both proteins, the composition of the solution will depend on the composition of the solid solution, but if the composition of the solid solution is fixed, the composition of the aqueous solution in equilibrium with it is also fixed. Lastly, if there are two solid phases the system again becomes invariant and the composition of the solution, when the temperature, pressure, and concentrations of acid and salt are fixed, is definite.

If additional components such as salts are present, each new component

TABLE I

Components	Phases	Degrees of freedom at constant temperature, pressure, and concentrations of acid and salt	Examples
4 = water, salt, acid, protein	1	1	A homogeneous solution
	2	0	A solid phase + saturated solution
	3	(−1)	Two solid phases + saturated solution. (Only possible at a particular concentration of acid or salt.)
5 = water, salt, acid, two proteins	1	2	A homogeneous solution
	2	1	One solid phase containing (*a*) a single protein, or (*b*) a solid solution of two proteins, in a solution of variable composition.
	3	0	Two solid phases in solution of constant composition

introduces a new degree of freedom; but if the concentrations of the new components are fixed, the degrees of freedom are unchanged.

The case of a solid solution of two proteins may be discussed in greater detail. Such a solution will be in equilibrium with an aqueous solution of *fixed composition*, but the ratio of the proteins in the aqueous solution may be either the same or different from that in the solid solution. In the first case an equilibrium solution can be formed by simple dissolution of the solid phase and therefore without any change in its composition. Therefore the solubility will be independent of the amount of the solid. In the second case the formation of the equilibrium solution will result in change in the composition of the solid, and as shown by Northrop and Kunitz (9) the effective solubility will then vary with the amount of the solid phase. The following is a simplified derivation of this conclusion, which does not depend on the particular assumptions previously made.

Suppose that the relation between the composition of the solid and that of the solution in equilibrium with it is as represented in Fig. 1, where X is the composition of the solid phase in equilibrium with a solution in which the ratio of the two proteins is represented by Y. In this illustration the proportion of A entering the solution is greater than that in the solid and the solid is therefore left with a smaller proportion of A. We will now consider the following cases:

1. If the quantity of solid is large compared with the amount dissolved, a solution of composition Y is obtained with a very small change in the composition of the solid and the solution obtained from the original crystals will effectively have the composition Y.

2. If the solid is nearly all dissolved, the composition of the solution must approximate to that of the original crystals and will be represented by Y'. The solid which is in equilibrium with this solution has the composition X'.

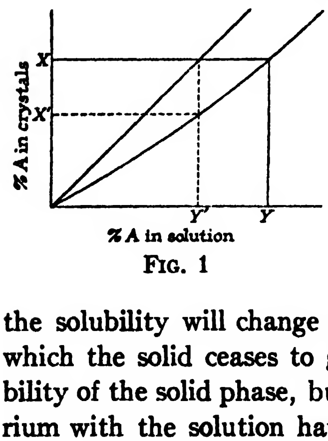


FIG. 1

If we add increasing amounts of the solid phase X to a definite quantity of the solvent, on the assumption that the solid phase can adjust itself to equilibrium with the solution, we should find that when excess of solid first appears it has the composition X' and a solubility which corresponds to the point Y'. As the excess of solid increases the solid phase remaining will change in composition from X' to X and the solubility will change correspondingly. It follows that the point at which the solid ceases to give a clear solution is not necessarily the solubility of the solid phase, but it will be the solubility of a solid in equilibrium with the solution having the same composition as the original solid phase. The amount of protein dissolved will be equal to the amount of protein added, up to the point at which solid first appears, but under these circumstances the solubility may then change as the excess of solid is increased until with a great excess of solid a constant value is obtained.

It is possible that the whole of the solid phase may not readily come to equilibrium with the solution and that the adjustments of composition may be confined to the surface layers of the crystals. In that case we may get the solubility which corresponds to a small residue of the solid while an appreciable amount of the solid remains.

The amount of protein present in a solution of definite acidity and salt concentration will therefore be independent of the amount of solid in contact with the solution, from the first appearance of turbidity, in the following cases:

1. The solid is a single phase. (*a*) A single pure protein, and (*b*) a solid solution of two proteins, which dissolve in the same proportion as they are present in the solid.

2. The solid consists of two distinct phases. (*a*) A mixture of two proteins, present in amounts proportional to their solubilities.

To distinguish cases 1 *b* and 2 *a* from 1 *a*, we may observe that it is unlikely that the solubilities of two proteins would be equally affected by a change of acidity and/or salt concentration. If the solvent is changed, it is therefore probable that in the case of the solid solution the two proteins will no longer dissolve in the same proportions and the solubility will then depend on the amount of the solid phase, as described above; while in the case of two distinct solid phases, increasing the amount of solvent will give rise to a point at which one solid has completely dissolved, and here the protein concentration becomes variable. Independence of the solubility of the amount of the solid phase in several solvents can therefore be taken as strong evidence that the material is a pure protein.

On the other hand, a single protein which is present in two distinct solid forms, *e.g.* amorphous and crystalline, may also give the solubility curve of a mixture. If equilibrium between the two solids and the solution were established, this would be a rare occurrence, since it can only be found in particular solvents in which it happens that the two forms have the same solubility. But the approach to equilibrium may be very slow and cases may be encountered in which the two solids persist for long periods although not in equilibrium and give rise to the solubility curve of a mixture. Such cases may be distinguished by adding sufficient solvent to dissolve one phase and examining the residue. If it gives identical solutions to the original solid, or can be changed into the other form, we are dealing with a case of polymorphism. To decide the nature of the material it is therefore necessary to examine not only the solubility curves in a number of solvents, but in case the curves of a mixture are obtained, to separate the solids and determine their nature.

Equilibration of the Protein with the Solvent

We must now consider how far the conditions of constant acidity and constant salt concentration can be satisfied with varying amounts of the solid protein. In the previous experiments of Kunitz and Northrop, it has been considered that this condition is satisfied by previously equilibrating the solid by washing with successive portions of the solvent in which the solubility is to be measured, until the solubility of the protein is constant. Then it is taken that the addition of varying amounts of the equilibrated

solid to the solvent will not produce variations of the acidity and salt concentration in the latter. A careful examination of the validity of this process is desirable. We can distinguish the following cases:

1. Suppose that the whole of the solid protein can come into equilibrium with the buffer. It is known that protein crystals contain considerable quantities of water and ions derived from the mother solution, and it has been found that appreciable differences of density (10), and of cell dimensions (11) occur according to the nature of the solution in which the crystals are suspended. It follows that the penetration of the salt solution in which the crystals are suspended occurs at least in some cases. In such a case the protein in the interior of the crystal may be expected to come into equilibrium with the solvent both in respect to acidity and salt concentration. On mixing a quantity of the solid with the buffer solution, the equilibrium finally reached will correspond with a pH intermediate between that of the buffer and the original protein. If the solution is poured away and a fresh quantity of the buffer solution added, a second equilibrium will be reached, and it seems evident that after successive treatments in this way the solid will approach a state in which further washings produce no change in the solid. The solubility will then be constant and will be independent of the ratio of the solid to liquid phases.

This procedure would fail only when the solubility of the protein is such that it dissolves completely before equilibrium is reached. This might be overcome by increasing the concentration of the buffer in such a way that the buffering power of a given quantity of solvent is increased, without increasing the solubility of the protein; or by using as the solvent a more concentrated salt solution in which the protein solubility is less.

2. The solid may not equilibrate at all with the buffer. The only function of successive washings would then be to remove the mother liquor adhering to the crystals. A given quantity of solvent would then always dissolve the same amount of protein; which will produce a constant change of pH. The change of pH produced by the dissolution of the protein would, however, depend on the pH of the protein preparation and the solubility will therefore probably depend on the nature of the solution in which the protein crystals were formed.

3. Suppose that the buffer solution reacts with the protein to form a new phase, *e.g.* a protein salt. It has been shown that two solid phases of a single protein can only co-exist with a solution in which one variable concentration, *e.g.* acidity, is fixed. The buffer solution will therefore be changed by the reaction to a composition which corresponds to the pH fixed by the protein solids. The addition of more buffer will produce more

of the protein salt, and only when the first solid phase has been completely converted into the second can the pH change and approach the value defined by the buffer solution. In this case it is evident that a stoichiometrical excess of the buffering substances is required to bring the protein into a state in which it can exist unchanged in contact with fresh portions of the buffer solution.

II. The Solubility Curves and Purity of Chymotrypsinogen

Chymotrypsinogen from beef pancreas was chosen for these experiments on account of its stability and ease of crystallization. Good solubility curves for this material have already been obtained by Kunitz and Northrop (12) using the activity, when converted into chymotrypsin, and the protein concentration as determined by turbidity measurements as measures of the solubility. In this work we have used the total nitrogen of the solution as the measure of the solubility; a more rigorous test since nitrogenous impurities of a non-protein character are included in the determined values.

Under these circumstances ammonium sulfate solutions cannot be used as the solvent and magnesium sulfate was substituted. The first experiments showed that when recrystallized seven or eight times from magnesium sulfate, this material gave quite good solubility curves, the solubility being practically independent of the amount of solid phase from the first appearance of turbidity, but the actual solubility varied somewhat from one preparation to another, and the residue left after dissolving part of the substance had an appreciably different solubility. This suggested that the material might be a solid solution and in an attempt to effect a separation a series of fractional crystallizations was carried out on twice crystallized material.

In this first series a solution of the protein was prepared at pH 4.0 and sufficient magnesium sulfate was added to cause the crystallization of about one-third of the total protein. The addition of more magnesium sulfate gave a further crop of crystals and the remainder was precipitated from a nearly saturated solution of the salt. This process was repeated on the fractions five times, the "less soluble" precipitate from one fraction being systematically united with the "more soluble" precipitate from the next. In this way three fractions were obtained, *A*, *B*, and *C*; *A* being obtained from the first precipitates and *B* and *C* from the middle and last fractions. The three fractions were crystallized twice from a pH 4.0, 0.4 saturated magnesium sulfate solution and the solubility curves were determined with this solvent. The curves obtained are shown in Fig. 2. Both *B* and *C*

gave very good solubility curves, the solubility being practically constant from the first appearance of turbidity, but the solubility of C is slightly

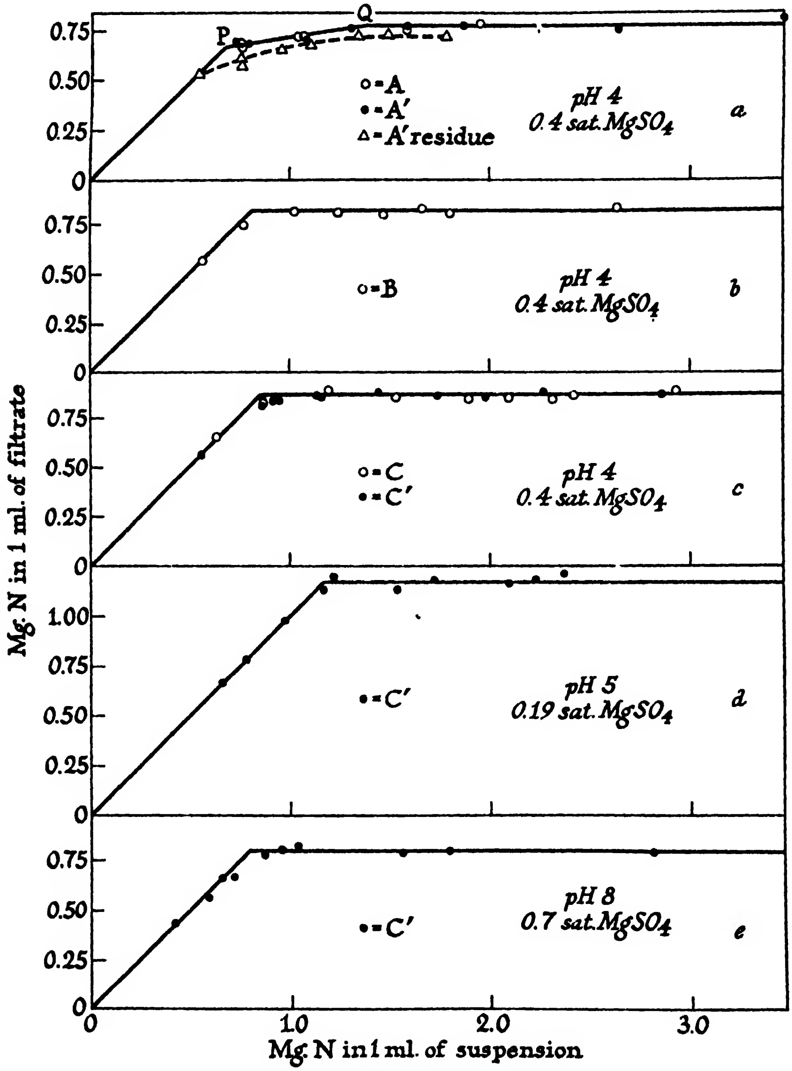


FIG. 2. Solubility curves of chymotrypsinogen fractions in various solvents

greater than that of B. The curve of A has a distinct break and the solubility continues to rise after the first appearance of turbidity at P to the region of Q, indicating that this material is complex.

The whole fractionation was repeated with new material in a somewhat simplified form in the course of which the middle fraction (B) was divided

between the two end fractions. In this way two fractions A', C' were finally obtained. It was found that the solubility curve of A' was identical with that of A and C' with the previous fraction C.

Since B and C' were obtained by adding more magnesium sulfate to a saturated solution of protein in approximately the same solvent as that in which the solubility was determined, it was possible that these fractions might contain two substances present in the same proportion as their solubility in this solvent. Solubility curves of C' were therefore deter-

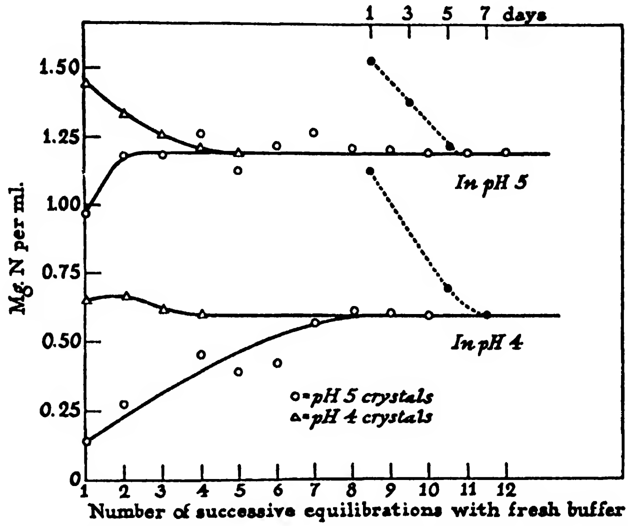


FIG. 3. Solubilities of crystals formed at pH 4.0 and pH 5.0. Triangles refer to crystals formed at pH 4.0, and open circles to crystals formed at pH 5.0. Closed circles represent successive concentrations in supersaturated solutions. The upper set of points is for solubilities in 0.19 saturated magnesium sulfate, pH 5.0 solvent; the lower set of points is for solubilities in 0.4 saturated magnesium sulfate, pH 4.0 solvent.

mined in two other solvents of pH 5.0 and 8.0, with suitable concentrations of magnesium sulfate (Fig. 2, *d* and *e*). In both cases a good solubility curve was obtained and since it is very unlikely that the ratio of the solubilities of two substances would be identical at three different pH's, this may be taken as strong evidence that C and C' are a single substance.

That a true equilibrium was established in these experiments was shown by the following facts.

1. The solubility of the crystals in a given solvent was independent of the pH of the solution in which the crystals were formed. Fig. 3 shows the solubilities of crystals formed in magnesium sulfate solutions at pH 4.0 and 5.0 in successive shakings with fresh portions of two distinct solvents. The

crystals made at both pH's ultimately reached the same solubility in either solvent.

2. The solubility of the crystals was independent of the concentration of magnesium sulfate in the solution in which they were formed.

Concentration of magnesium sulfate	0.35 saturated	0.4 saturated	0.5 saturated
Solubility in approximately 0.4 saturated magnesium sulfate at pH 4.0 (mg. N/ml.)	1.01	0.98	0.97

3. The same equilibrium was reached from supersaturated as from undersaturated solutions. The supersaturated solutions were prepared by cooling a saturated solution with excess of the crystals. More dissolves at

TABLE II

Characteristics of Products of Fractionation

	A'	C'
Non-protein nitrogen, *per cent*	4 0	0.2
Milk clotting activity per mg. nitrogen. Average of 5	10.5	10.6
Hemoglobin activity per mg. nitrogen*	0.61×10^{-3}	0.62×10^{-3}
Velocity constant of activation by a trypsin solution, $hrs.^{-1}$	0.83	0.83
Migration velocity, $cm.^2$/sec. volt (cathode compartment)	0.675	0.667

*For these measurements I am indebted to Miss M. McDonald. For method see Anson (14).

the lower temperature and when the temperature is raised again a supersaturated solution is obtained. After several days the protein concentration returns to the original value (broken lines of Fig. 3).

The material A, which has a complex solubility curve is less soluble than B and the solubility decreases on repeated extractions. It is probable that this material contains some substance which is precipitated at the smaller salt concentration and which forms a solid solution with the chymotrypsinogen.

Apart from the solubility curves and a rather greater proportion of nonprotein nitrogen in A', no significant difference between the fractions A' and C' could be detected. When converted by trypsin into chymotrypsin, they had practically the same activities per milligram nitrogen, as measured both by milk clotting and by the digestion of hemoglobin, and the velocity constants of the activation process were the same within the experimental error (see Table II). Since the accuracy of these measurements is of the order of 5 per cent it can be concluded that the impurity in A and A',

which is not present in C, does not contribute more than 5 per cent of the total nitrogen, unless it is also capable of activation. It must be remembered that if the molecular weight of the impurity is low the molar fraction may be appreciable and sufficient to produce a significant change of solubility.

The impurity in this fraction is probably present in solution in the protein crystals. If it were a separate phase the solid residue in the region PQ, in which the solutions are saturated with the main constituent but the subsidiary constituent continues to dissolve (Fig. 2 *a*), should consist of the pure protein. Some of this residue was collected and its solubility curve, shown by the dashed line in Fig. 2 *a*, indicates a greater concentration of the impurity in the residue; as might be expected for a solid solution. The dialysis of solutions of the fraction A' caused a small decrease in the amount of non-protein nitrogen, but had no significant effect on the solubility curve.

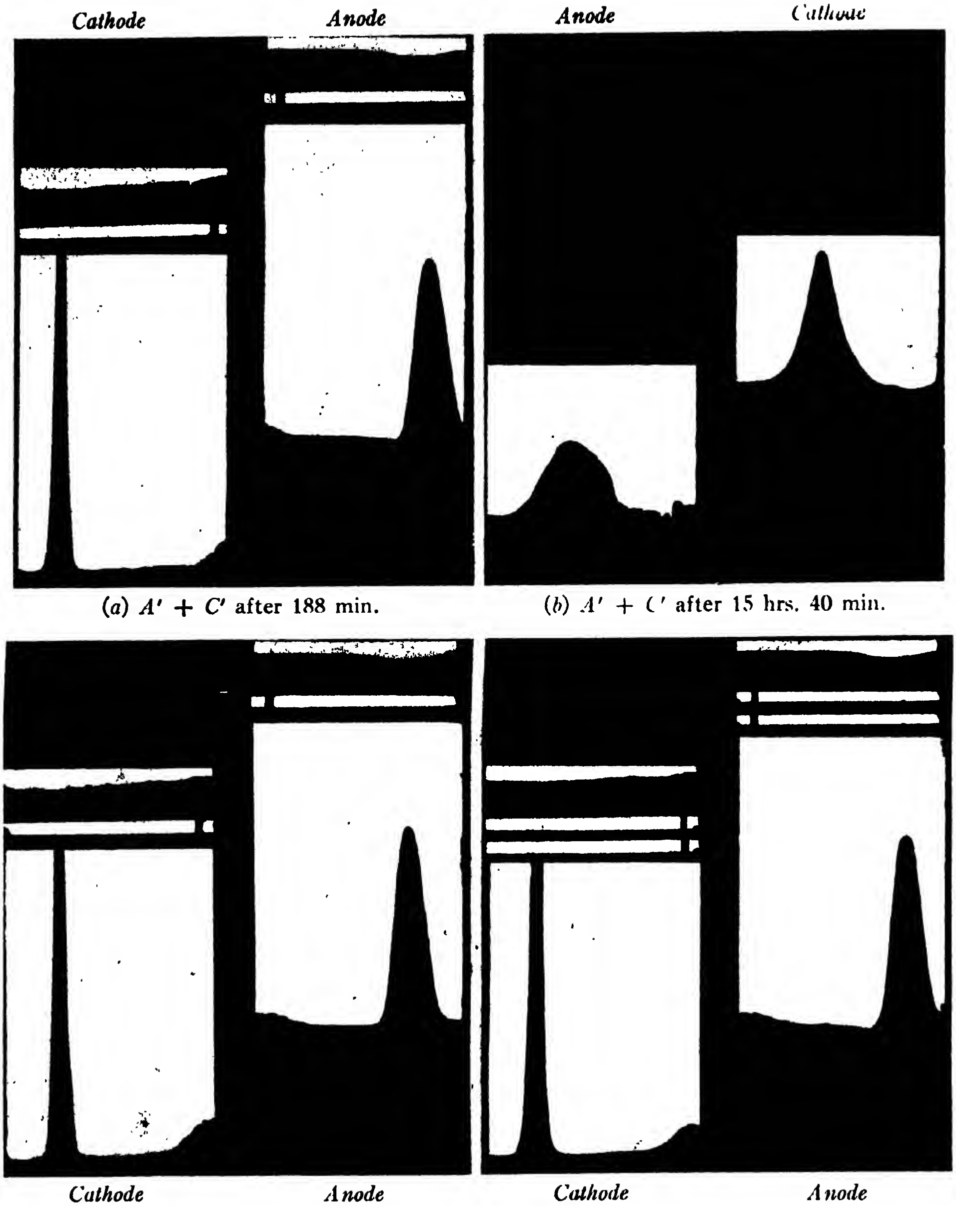
The electrophoresis of solutions A' and C' and of a mixture of A' and C' in a M/15 citrate-hydrochloric acid buffer solution at pH 3.0 after equilibration by dialysis with a large quantity of this buffer, was kindly examined by Dr. Alexandre Rothen of The Rockefeller Institute for Medical Research in New York. The electrophoretic patterns obtained by him, using Longsworth's schlieren scanning technique, are shown in Fig. 4.

In all cases only a single moving boundary was observed and the cathodic boundaries in every case were extremely sharp. The mixture of A' and C' showed no signs of any resolution even after more than 15 hours migration. The migration velocities were approximately the same in all solutions, the small differences being attributable to slight variations of the pH in the dialyzed solutions.

We conclude that no genuine fractionation of the protein has been achieved, but a small quantity of impurity has been concentrated in the fractions first precipitated. This is insufficient in quantity to produce any appreciable difference in the enzymatic properties and it does not produce a visible boundary in the electrophoretic pattern. But if the molecular weight of the impurity is comparatively low, its molar fraction in the crystals may be sufficient to produce the observed diminution of solubility of the protein and the break in the solubility curve. The final fractions satisfy every test of a pure substance which has been applied.

EXPERIMENTAL METHODS

Preparation of the Material and Methods of Crystallization.—The chymotrypsinogen was prepared from beef pancreas by the method described by Kunitz and Northrop (13). It was recrystallized from magnesium sulfate by dissolving in 2.5 volumes of 0.2 M acetic acid and then sufficient sodium acetate was added to produce a buffer of pH 4.0



(a) A' + C' after 188 min.

(b) A' + C' after 15 hrs. 40 min.

(c) A' after 151 min.

(d) C' after 162 min.

FIG. 4. Electrophoresis patterns of fractions A' and C' and mixtures of both (Dr. A. Rothen).

Solution A'; pH 2.98, velocity in cathode compartment 0.675×10^{-4} cm.^{2}/sec. volt; gradient 4.09 volt/cm.

Solution C'; pH 2.98, velocity in cathode compartment 0.667×10^{-4} cm.^{2}/sec. volt; gradient 4.09 volt/cm.

Solution A' + C': pH 3.0, velocity in cathode compartment 0.608×10^{-4} cm.^{2}/sec. volt; gradient 4.09 volt/cm. for 188 minutes, 2.04 volt/cm. for additional time.

(22.6 ml. 0.2 M sodium acetate to 100 ml. 0.2 M acetic acid), followed by 2 volumes of saturated magnesium sulfate. Nearly the whole of the protein crystallized out in the course of a few hours. In the fractionations the protein was dissolved in 2 volumes of acetic acid and the same proportion of sodium acetate; then sufficient magnesium sulfate was added to bring the concentration to one-third saturation. The crystals formed were filtered off and the magnesium sulfate concentration was raised to about 0.45 saturation, when a further crop of crystals was obtained. The remaining protein was precipitated by adding solid magnesium sulfate to saturation. In Series II, after the first crop of crystals, the magnesium sulfate concentration was raised at once to about 0.7 saturation.

Determination of the Solubility.—The protein was placed in a test tube with a marble or ball-bearing and the tube was completely filled with the solvent up to the rubber stopper. It was rocked in a thermostat at 19.5°C. ± 0.1° for 2 days. The tubes were now centrifuged and a portion of the clear supernatant liquid was carefully removed by pipette for analysis. These operations were carried out in a constant temperature room at 19.5°C. ± 0.5°. The total nitrogen in the liquid was determined by a semi-micro Kjeldahl method using 1 ml. samples. The material was shaken with fresh portions of the solvent until two successive solubilities were the same and then a "solubility curve" was obtained by distributing the suspension in varying amounts among tubes filled with solvent.

SUMMARY

1. The conditions under which the phase rule may be applied to systems containing proteins are formulated.

2. An attempt was made to fractionate chymotrypsinogen, by crystallization in stages with increasing concentration of magnesium sulfate. No significant fractionation of the protein was achieved, but a small amount of impurity which affects the solubility, while having little influence on other properties of the material, was concentrated in the fractions first precipitated.

3. The solubility of the final fraction was independent of the amount of the saturating solid, from the first appearance of a solid phase, in solvents of three different pH's. The solubility was independent of the environment in which the crystals were formed (within the limits in which crystallization can be carried out) and the same value was reached from the supersaturated as from the undersaturated side. This material, therefore, conforms closely with the phase rule criteria of a pure protein.

The author wishes to express his thanks to Dr. John H. Northrop for granting him the facilities of his laboratory and for much valuable advice.

LITERATURE

1. Hardy, W. B., *J. Physiol.*, 1905, **33**, 251.
2. Mellanby, J., *J. Physiol.*, 1905, **33**, 338.
3. Sörensen, S. P. L., and Höyrup, M., *Compt.-rend. trav. Lab. Carlsberg*, 1917, **12**, 213.

4. Sörensen, S. P. L., *Compt.-rend. trav. Lab. Carlsberg*, 1923, **15**, No. 11; 1926, **16**, No. 8; *J. Am. Chem. Soc.*, 1925, **47**, 457.
5. Sörensen, S. P. L., *Compt-rend. trav. Lab. Carlsberg*, 1930, **18**, No. 5.
6. *E.g.*, horse carboxy-hemoglobin, Cohn, E. J., and Prentiss, A. M., *J. Gen. Physiol.*, 1927, **8**, 619; Sörensen, S. P. L., and Sörensen, M., *Compt.-rend. trav. Lab. Carlsberg*, 1933, **19**, No. 11; lactoglobulin, Palmer, A. H., *J. Biol. Chem.*, 1934, **104**, 359; pepsin, Northrop, J. H., *J. Gen. Physiol.*, 1930, **13**, 739; trypsin, Northrop, J. H., and Kunitz, M., *J. Gen. Physiol.*, 1932, **16**, 267.
7. Northrop, J. H., and Kunitz, M., *J. Gen. Physiol.*, 1930, **13**, 781.
8. Kunitz, M., and Northrop, J. H., in Cold Spring Harbor symposia on quantitative biology, Cold Spring Harbor, Long Island Biological Association, 1938, **6**, 325; *Compt.-rend. trav. Lab. Carlsberg*, 1938, **22**, 288.
9. Northrop, J. H., and Kunitz, M., *J. Gen. Physiol.*, 1930, **13**, 781.
10. Adair, G. S., and Adair, M. E., *Proc. Roy. Soc. London, Series B*, 1936, **120**, 422. Crowfoot, D., *Proc. Roy. Soc. London, Series B*, 1938, **64**, 580.
11. Crowfoot, D., and Riley, D., *Nature*, 1939, **144**, 1011.
12. Kunitz, M., and Northrop, J. H., *Compt.-rend. trav. Lab. Carlsberg*, 1938, **22**, série chimique, 288.
13. Kunitz, M., and Northrop, J. H., *J. Gen. Physiol.*, 1935, **18**, 433.
14. Anson, M. L., *J. Gen. Physiol.*, 1938, **22**, 79.

MOLECULAR WEIGHT AND ELECTROPHORESIS OF CRYSTALLINE RIBONUCLEASE

By ALEXANDRE ROTHEN

(*From the Laboratories of The Rockefeller Institute for Medical Research*)

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The present paper describes the results of ultracentrifugation, diffusion, and electrophoresis of crystalline ribonuclease whose methods of preparation and properties have been reported by Kunitz (1).[1]

Electrophoresis Studies.—Experiments were carried out from pH 4 to pH 10 in a Tiselius apparatus at 0° using the Longsworth scanning method for the observation of the boundaries (2). 25 to 30 ml. of solutions containing 1 per cent of three times crystallized ribonuclease were made up in appropriate buffers and dialyzed in collodion bags for several days at 5°C. against 2 liters of the corresponding buffer solution. The outside buffer solutions were used to fill the upper compartments of the Tiselius apparatus.

The results have been summarized in Fig. 1 which represents the mobilities of the sharper boundaries as a function of pH. Phosphate buffers were used from pH 6.8 to pH 8.8 and acetate buffer at pH 4.1, the ionic strength being $\simeq 0.055$. Two experiments were made in borate buffer at pH 8.9 and pH 10.1, ionic strength $\simeq 0.03$.

The mobilities observed in the two latter cases were considerably displaced. The following values were found: $u = +1.4 \times 10^{-5}$ at pH 8.9 and $u = -1.4 \times 10^{-5}$ at pH 10.1. It is apparent from Fig. 1 that *ribonuclease has a very high isoelectric point at about pH 7.8.*

The electrophoretic patterns indicated the presence of only one moving component. The boundaries showed the phenomenon of reversible boundary spreading to a considerable extent. The boundaries moving towards the cathode were much sharper than the boundaries moving towards the anode. A few patterns have been reproduced in Fig. 2. The boundaries at pH 4.1, 7.8, and 8.9 can be seen in Fig. 2 a, 2 b, and 2 c respectively.

Diffusion Measurements.—Diffusion measurements were carried out at 25° in the apparatus used for the electrophoresis experiments. A 1 per

[1] I am greatly indebted to Dr. Kunitz who kindly prepared the solutions of ribonuclease used in these investigations.

cent solution of ribonuclease in 0.5 M $(NH_4)_2 SO_4$, pH 5.8 was used. This solution had been dialyzed against a large volume of 0.5 M $(NH_4)_2SO_4$ which was used to fill the upper compartments of the cell into which the ribonuclease was diffusing. The results can be seen in Fig. 3 which represents the appearance of the boundary 53, 193, 373, 663, 1337, and 1842 minutes after its formation. The following formula was used for the calculation of the diffusion constant:

$$D = \frac{S^2}{4\pi t H^2 \text{ max.}}$$

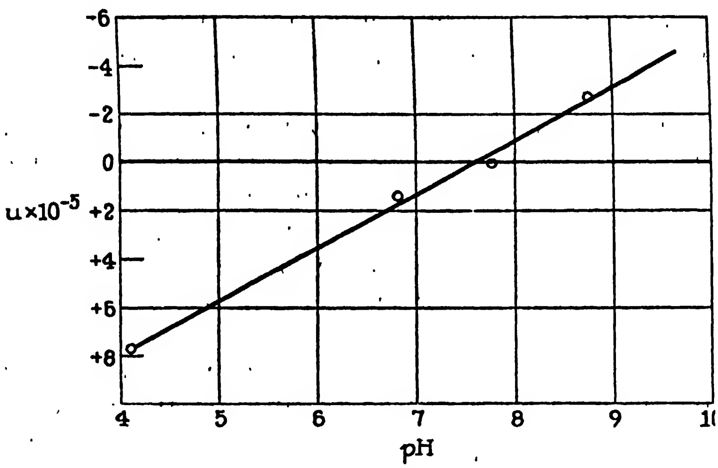

FIG. 1. Mobilities in cm.²/sec.volt of ribonuclease

where D is the diffusion constant in cm.²/sec., S the diffusion area in cm.², t time in seconds and $H_{max.}$ the maximum height of the curve in cm. (3). Since the magnification factor of the camera was unity, and since the terms S and H are both squared, the absolute magnitude of the deflection of the rays depending on the geometry of the apparatus has no importance. The only assumption is that the refractive index varies linearly with the concentration (within the range 0 to 1 per cent). The diffusion area should stay constant during the entire experiment. As can be seen from Table I, the value found for the areas is reasonably constant.[2]

From these data the value of the diffusion constant was computed to be $D^{25} = 1.36 \times 10^{-6}$ (in 0.5 M $(NH_4)_2 SO_4$).

[2] The diffusing areas after 53′ and 193′ were too large. This increase in area might be due partly to spherical aberration of the camera objective which might play a part when the extreme rim of the lens was used in the focusing of the extreme deviated rays.

Ultracentrifuge Studies.—The apparatus used was an air-driven centrifuge of the turret type described by Bauer and Pickels (4). Three methods were used to follow the course of sedimentation: the light absorption method for which a resonance mercury arc lamp served as source of light, the scale method of Lamm, and the "schlieren method" as used by Svensson in electrophoretic studies (5). The speed of the centrifuge was measured by a 631 A Strobotac built by the General Radio Company.

Rate of Sedimentation.—On account of the relatively low molecular weight of this enzyme, long time intervals were needed to obtain a sufficient displacement of the boundary. During that time a considerable amount of diffusion took place, making it difficult to determine accurately the maxima of the displacement curves obtained by the scale or schlieren methods as well as the position of 50 per cent concentration range when the absorption method was used. Only one moving component was observed, and the symmetry of the curves (absorption, schlieren, or "scale" curves) indicated the fair homogeneity of the material. A schlieren diagram of the last experiment reported in Table II can be seen in Fig. 4. The abscissa represents the length of the cell, the material sedimenting from right to left. The ordinate is the displacement measuring the refraction gradient. The possibility of superimposing on a single plate the patterns obtained during the course of sedimentation is a distinct advantage of the method since it permits an easier computation of the rate of sedimentation. The interval between the first and second, as well as between the second and third curves was 1 hour, whereas between the third and fourth curves it was 2 hours.

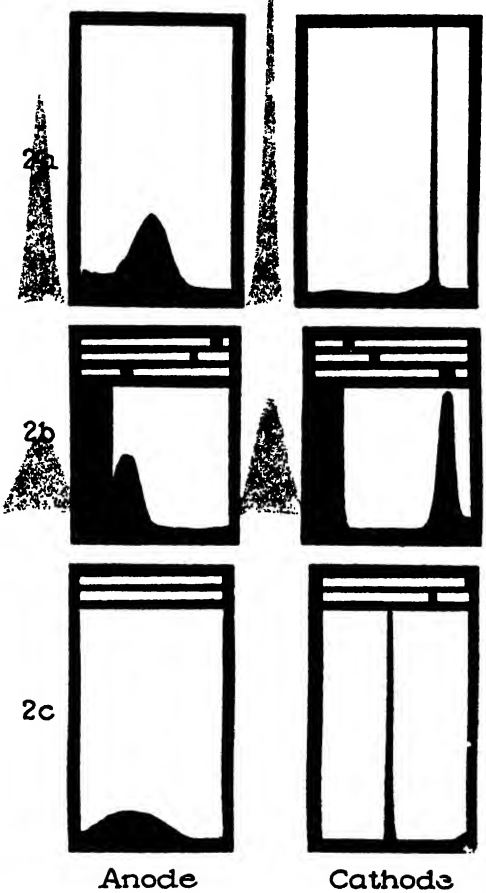

FIG. 2. Electrophoretic patterns of ribonuclease.

The microphotometer curves obtained by the absorption method for run 2 of Table II are reproduced in Fig. 5. The time of the first curve

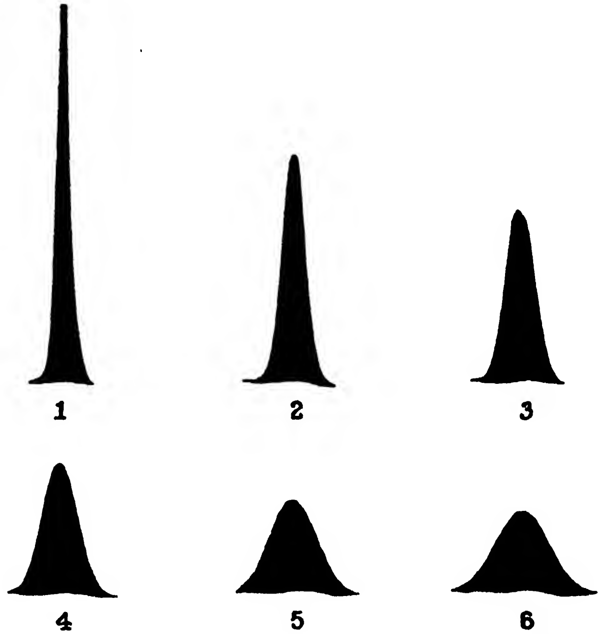

FIG. 3. Diffusion patterns of ribonuclease

TABLE I

Time, *min.*	373	663	1337	1842
Area	2.23_5	2.23_5	2.22	2.22
Maximum height	3.40	2.65	1.89	1.61

TABLE II

d_4^{25}	η^{25*}	Thickness cell	Speed	Total time	Mean temperature	$s_{20}\times 10^{13}$			$s^0_{20}\times 10^{13}$		
						Absorption	Scale	Schlieren	Absorption	Scale	Schlieren
		mm.	R.P.M.	*hrs.*	°C.						
1.1160	1.527	3	54000	5½	26	1.12	1.01	—	2.35	2.12	—
1.0366	1.145	3	54000	5	27.2	1.91	1.88	—	2.41	2.37	—
1.0366	1.145	6	57600	4	27.8	—	1.79	1.87	—	2.26	2.37
1.0366	1.145	6	57600	4½	26.5	—	1.85	1.86	—	2.33	2.35

* η^{25} is the viscosity referred to that of water at 25° as unity.

being zero, that of the second, third, fourth, fifth, sixth, and seventh curves is 1, 2, 2½, 3, 4, and 5 hours, respectively.

Different preparations of crystalline ribonuclease were used. No difference in the rate of sedimentation could be detected in material recrystallized from ammonium sulfate solution or from dilute alcohol. Solutions of 1 per cent ribonuclease and cells 3 and 6 mm. thick and 15 mm. long were used. The distance from the center of rotation to the middle of the cell

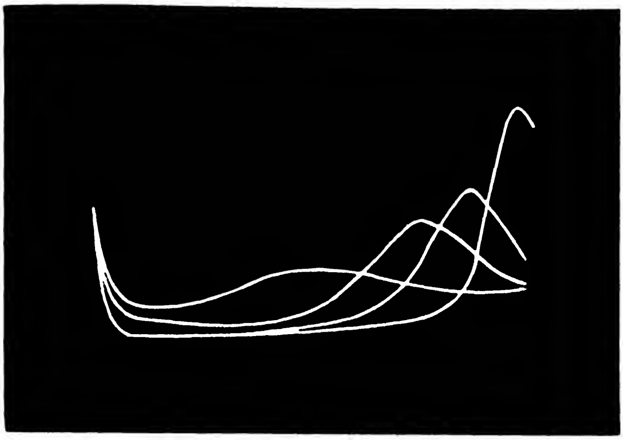

FIG. 4. Schlieren diagram of ribonuclease

FIG. 5. Microphotometer curves. Absorption method

was 6.5 cm. The results have been summarized in Table II. The medium was a solution of 0.4 saturated ammonium sulfate in 0.1 M acetate buffer pH 4.1 for the first run and a solution of 0.5 M $(NH_4)_2SO_4$ for the others. The constants of sedimentation, s_{25}°, included in the last column, have been calculated from the usual correction formula

$$s_{25}^{\circ} = s_t \frac{\eta_t}{\eta_{25}^{\circ}} \frac{1 - V_{25}\rho_{25}^{\circ}}{1 - V_t\rho_t}$$

where η_{25}° is the viscosity and ρ_{25}° the density of water at 25° and η_t and ρ_t the same constants for the medium used in the experiment. V is the specific volume of the protein as determined below. The observed rate of sedimentation of the first experiment reported in Table II is small on

account of high viscosity and high density. However, this value reduced to water agrees as well as can be expected with the values calculated from the other experiments. As can be seen, no systematic deviation could be observed between the three methods of observation of the boundaries.

At the highest speed (57,600 R.P.M.) an increase of temperature of the rotor of $\simeq 1.2°$ per hour was observed when the vacuum was as good as possible; *i.e.*, 10^{-5} mm. Hg. This increase was mainly due to heat generated by friction of the residual air on the rotor. When computing the rate of sedimentation, the mean temperature between each successive position of the boundary was taken into account.

Specific Volume. The specific volume was calculated from accurate determination of densities of solutions of known concentration. The following determinations were made:

Concentration	4.89	per cent in water,	$d_4^{25} = 1.0114$,*	specific volume $V_{25} = 0.709$
"	4.89	" " " "	$d_4^{20} = 1.0127_5$	" " $V_{25} = 0.704$
"	2.443	" " " "	$d_4^{25} = 1.0043$	" " $V_{25} = 0.707$
"	2.375	" " " "	0.5 M $(NH_4)_2SO_4$, $d^{25} = 1.0405$	specific volume $V_{25} = 0.710$

* Densities are based on weights in vacuo.

The value 0.709 was used for calculation. It should be noted that it is a very low value compared to that found for most proteins with the exception of cytochrome.

Molecular Weight from Rate of Sedimentation. Taking $s^{25} = 1.85 \times 10^{-13}$ (in 0.5 M $(NH_4)_2SO_4$) and $D = 1.36 \times 10^{-6}$ (in 0.5 M $(NH_4)_2SO_4$), the molecular weight according to the expression $M = \dfrac{RTs}{D(1 - V\rho)}$ becomes $M = 12{,}700$.

Molecular Weight from Sedimentation Equilibrium.—A sedimentation equilibrium run was made in 0.5 M $(NH_2)_2SO_4$ solution in a cell 6 mm. thick and 15 mm. long. The speed was 14,400 R.P.M. and the temperature 21.8°. The scale method was used for the determination of the distribution of the refraction gradients with a scale distance of 10 cm. Total time of centrifugation was 66 hours.

Fig. 6 summarizes the results. The curve represents the scale displacement as a function of the distance from the center of rotation from 6.466 cm. to 7.047 cm.[3] Open circles represent measurements after $59\frac{1}{2}$ hours;

[3] The difference $7.047 - 6.466 = 0.581$ corresponds to 6 divisions of the diagram because the latter are a measure of the comparator readings which have to be reduced to cell distances by the factor 0.968, hence $\dfrac{0.581}{0.968} = 0.6$.

solid triangles, measurements after 66 hours. No systematic differences can be observed between the two sets of values and it can be concluded that equilibrium had been reached.

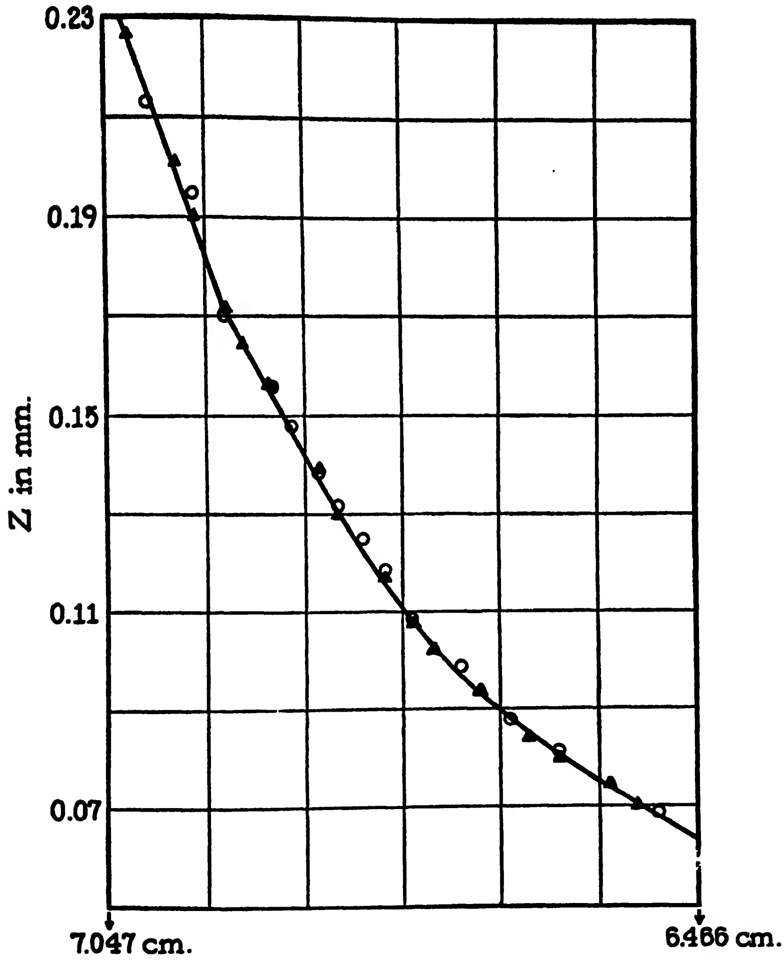

FIG. 6. Sedimentation equilibrium curve by the scale method

The following formula was used for the calculation of molecular weight:

$$M = \frac{2RT \ln [Z_{x_1}x_2/Z_{x_2}x_1]}{(1 - V\rho)(x_2^2 - x_1^2)}$$

where Z_x is the scale displacement at distance x from the center of rotation. From four pairs of values of Z_x and corresponding x read on the curves, the following values for M were obtained: 12,000, 12,900, 13,400,

13,700, with a mean value $M = 13{,}000$ in good agreement with the value obtained from rate of sedimentation and diffusion data. Kunitz (1) found $M = 15{,}000 \pm 1000$ from osmotic pressure measurements.

There was observed a regular shift in the molecular weight depending on which pair of values was chosen for the calculation. Values corresponding to positions near the bottom of the cell gave higher values than those near the meniscus.

This low value found for the molecular weight of a protein molecule shows how much caution should be exerted before accepting the idea of a universal protein building-stone of a weight of 17,600 (6).

Dissymmetry Factor.—Since the molecular weight has been determined from parameters which do not include the assumption of Stokes' law (equilibrium measurements or sedimentation plus diffusion measurements) it is possible to calculate the dissymmetry factor f/f_0 from the formula

$$\frac{f}{f_0} = \frac{RT}{6\pi\eta ND}\left[\frac{4\pi ND(1 - V\rho)}{3VRTs}\right]^{\frac{1}{3}}$$

where all symbols have their usual significance, η is the viscosity in poises of the medium (not the solution) and N the Avogadro number. Introducing the numerical values, one finds $\frac{f}{f_0} = 1.04$. This low value shows the high degree of symmetry of the shape of the molecule of ribonuclease, the most symmetric of all proteins investigated. With the aid of the formula of Herzog, Illig, and Kudar and of Perrin, the ratio of the two main axes of the molecules is calculated to be about 2.

SUMMARY

Electrophoretic studies on purified crystalline ribonuclease showed the absence of any impurities differing in mobility from the bulk of material.

The isoelectric point of ribonuclease was found by electrophoresis to be at about pH 7.8.

Ultracentrifuge studies indicated fair homogeneity of ribonuclease in solution. Only one moving component has been observed.

The molecular weight of ribonuclease was found to be 12,700 from rate of sedimentation ($s^{25} = 1.85 \times 10^{-13}$ in 0.5 M $(NH_4)_2SO_4$) and diffusion measurement ($D = 1.36 \times 10^{-6}$ in 0.5 M $(NH_4)_2SO_4$), in good agreement with the average value of 13,000 found from equilibrium measurements. This low value for the molecular weight of a protein would seem to discredit the value 17,600 as representing a universal unit weight for proteins in general.

The specific volume was found to be 0.709.

The dissymmetry factor $\frac{f}{f_0}$ was found equal to 1.04.

REFERENCES

1. Kunitz, M., *J. Gen. Physiol.*, 1940–41, **24**, 15.
2. Longsworth, L. G., *J. Am. Chem. Soc.*, 1939, **61**, 529.
3. Polson, A., *Kolloid-Z.*, 1939, **87**, 149.
4. Svedberg, T., The ultracentrifuge, Oxford University Press, 1940, 191.
5. Svensson, H., *Kolloid-Z.*, 1939, **87**, 183.
6. Svedberg, T., The ultracentrifuge, Oxford University Press, 1940, 406.

FRACTIONATION OF PEPSIN

I. Isolation of Crystalline Pepsin of Constant Activity and Solubility from Pepsinogen or Commercial Pepsin Preparations. II. Preparation of a Less Soluble Fraction. III. Solubility Curves of Mixtures of the Soluble and Insoluble Fractions. IV. Preparation of Highly Active Pepsin from Pepsinogen

By ROGER M. HERRIOTT, V. DESREUX,* and JOHN H. NORTHROP

(*From the Laboratories of The Rockefeller Institute for Medical Research, Princeton, New Jersey*)

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Fractionation of Pepsin

The experimental evidence in favor of the protein nature of enzymes has accumulated to such an extent that this view is now widely accepted. The question as to whether the enzyme preparations are pure proteins is, however, much less certain. The problem is complicated by the difficulty of defining a pure protein. If homogeneity in the ultracentrifuge or electrophoresis cell is considered as a sufficient test of purity then many proteins, including several enzymes, have been obtained in pure form. If constant solubility be considered as the criterion of purity then no protein, with the possible exception of trypsin (Kunitz, 1938) and chymotrypsinogen (Butler, 1940; Kunitz and Northrop, 1938) has been prepared in pure form. The earlier results with pepsin may be taken as an example. Thus this protein is homogeneous in the ultracentrifuge (Philpot, 1935) and in the electrophoresis cell (Tiselius, 1938; Herriott, Desreux, and Northrop, 1940). The earlier preparations of Northrop (1930) showed constant solubility in certain solvents but not in others. Steinhardt was unable to obtain a sample of constant solubility in dilute acid. In addition, Northrop (1933) found that some samples of crystalline pepsin contained nearly 50 per cent inert protein and that all samples contain more or less of another enzyme especially active on gelatin (Northrop, 1931). Herriott (1938) found that pepsin prepared from different sources varied in specific activity. Holter (1931), and Dyckerhoff and Tewes (1933) also found slight variations in

* Fellow of the Belgian American Foundation.

activity. Desreux and Herriott (1939) have recently shown that these variable results are probably due to the fact that crude pepsin preparations may contain more than one active component which were not separable by the original method of purification and which differ in solubility. It became necessary, therefore, to develop a method of separating and isolating one or more of these proteins.

Desreux and Herriott (1939) showed that there were probably at least two active proteins present, a more soluble one which has an activity of about 0.33 $[\text{P.U.}]^{\text{Hb}}_{\text{P.N.}}$ and a less soluble one having an activity of about 0.2 $[\text{P.U.}]^{\text{Hb}}_{\text{P.N.}}$ or less.

The more active and more soluble component has been isolated by repeated extraction with 0.6 saturated magnesium sulfate at pH 5.0 and crystallized. It has constant activity and constant solubility in several solvents. It appears to be at least as pure as any other known protein with the exception of chymotrypsinogen.

A more insoluble fraction has been prepared by repeated precipitation with 0.45 saturated magnesium sulfate at pH 5.0 which has about two-thirds the activity of the soluble fraction as measured by hemoglobin, but the same or slightly higher activity as measured by clotting of milk, digestion of casein, or casein or gelatin viscosity changes. This preparation does not have constant solubility and is undoubtedly still a solid solution of two or more proteins. When mixed with the pure soluble fraction variable solubility curves are obtained which resemble those of the original crude pepsin preparation.

A third fraction has been obtained by dialysis in salt free solution at pH 4.0 which resembles this insoluble fraction (Desreux, unpublished experiments).

A further fraction having a specific activity of about 0.6 $[\text{P.U.}]^{\text{Hb}}_{\text{P.N.}}$ as measured by hemoglobin digestion has been obtained in small amounts from certain samples of pepsinogen. This fraction is extremely unstable and does not appear in commercial preparations.

I. Isolation of Crystalline Pepsin of Constant Activity and Constant Solubility in Certain Solvents

Desreux and Herriott (1939) have shown that the solubility curves for crude pepsin preparations demonstrate the presence of at least two proteins. These preliminary experiments indicated that the proteins were present as distinct solid phases; *i.e.*, the crude preparation is a mixture. The present experiments show conclusively, however, that this is not the case but that the proteins are present as one solid phase of varying composition; *i.e.*, a

solid solution. Application of Raoult's law to such systems (Northrop and Kunitz, 1930) predicts that the residue left when nearly all the material has dissolved will contain more of the insoluble components than the original sample while the fraction dissolved when only a small amount of solid is dissolved will contain more of the soluble components. The solubility curve, therefore, not only indicates the composition of the original material but also furnishes a method of separation of the components. This method is much more delicate than the usual method of fractionation by which the salt concentration is varied, instead of the protein concentration.

The fractionation was found to be more complete the more alkaline the solution and, as predicted, the smaller the fraction dissolved. It was eventually found that a nearly complete separation could be obtained by extracting the crude protein with a large volume of a solution of 0.6 or 0.65 saturated magnesium sulfate in 0.2 M pH 5.0 acetate. The pH is that of the maximum stability of pepsin. The method has been found satisfactory and reproducible with three preparations of pepsin from pepsinogen and with eight different preparations of Cudahy U.S.P. 1:10,000 soluble pepsin. One preparation from pepsinogen which had been stored at −13°C. for 2 years did not give a complete separation by this method and some of the commercial preparations required more repeated extraction than others.

The experimental details of two preparations of the material from pepsinogen are shown in Tables I and II. The solubility curves of the various fractions, in the amorphous form in (0.5 saturated magnesium sulfate, 0.05 M pH 4.6 acetate) are shown in Fig. 1.

It will be seen that there is little change in the specific activity but that the solubility of the extracted protein is much more nearly constant than that of the original preparation. The residue after extraction is less soluble and also inhomogeneous (Fig. 1 B). The soluble fraction after crystallization gives the theoretical curve for a protein containing only one component. Further extraction of this preparation causes no significant change in the solubility curves. The curves for the original sample, the portion dissolved, and the residue are the same (Fig. 1A).

The solubility of the fraction extracted increases with each step in the purification while the solubility of the residue decreases. This result confirms the conclusion that the original preparation is a solid solution rather than a mixture since in the latter case the saturated solution in the presence of excess solid must contain all the components and repetition of the solubility determination on this fraction must therefore yield the same value as that obtained from the original preparation.

The solubility of the crude material is intermediate between that of the

TABLE I

Preparation of Samples 13-2, 13-5, 13-8, 13-10, 13-12, and 13-13

	No.	Quantity		P.N./ml.	Total P.N.	[P.U.]Hb/mg. P.N.
				mg.	*mg.*	
150 gm. purified amorphous pepsinogen-ammonium sulfate filter cake dissolved in 750 ml. M/10 dipotassium phosphate and dialyzed 24 hrs. against tap water (pH 6.5)	1	1000	ml.	6	6,000	
No. 1 plus 850 ml. water plus 125 ml. N/1 hydrochloric acid plus 75 ml. saturated magnesium sulfate; stirred and let stand at 25°C. for 10 min. (pH 1.5–2.0). 2800 ml. saturated magnesium sulfate added slowly with constant stirring and filtered on hardened paper. Filtrate discarded. Precipitate	2	110	gm.		4,000	0.30
Residue No. 2 plus 5 liters (0.65 saturated magnesium sulfate, 0.2 M pH 5.0 acetate solvent A). Stirred as a uniform suspension for 3 hrs., filtered. Precipitate	3					
Filtrate	4	4800	ml.	0.31	1,500	0.34
Precipitate No. 3 plus 5 liters solvent A stirred 3 hrs., filtered. Precipitate	5	30	gm.			
Filtrate	6	5000	ml.	0.24	1,200	0.33
Filtrate No. 4 plus filtrate No. 6	7	9800	ml.	0.28	2,700	0.33
500 ml. No. 7 + 75 gm. solid magnesium sulfate dissolved; then 10 ml. 4 M acetic acid; pH = 4.6, filter. Filtrate discarded. Precipitate	8	3.3	gm.			
Remainder of solution No. 7 + 1400 gm. solid magnesium sulfate* stirred and 350 ml. 5 N sulfuric acid added (pH 2.0), filtered in ice box and washed once with 100 ml. cold N/50 sulfuric acid and filtered dry. Precipitate = 60 gm. Crystallized, see Table I *a*. Crystal cake filtered. Filtrate	9	52	ml.	4.8	250	0.26
Crystal cake washed 5 times on the funnel with 10 ml. 0.55 saturated magnesium sulfate, M/10 pH 4.0 acetate; washings discarded. Crystal cake	10	39	gm.		1300	0.30
10 gm. No. 10 + 100 ml. of solvent B (0.4 saturated magnesium sulfate, M/1 pH 4.6 acetate) stirred at 25°C. rapidly without foaming for 1.5 hrs. Centrifuged and washed once with 10 ml. solvent B. Combined filtrate and washings	11	110	ml.	2.2	240	0.29
Precipitate	12	3–4	gm.		120	0.30
No. 11 + equal volume of saturated magnesium sulfate, filter; filtrate discarded. Precipitate	13					

* In this and all subsequent tables solid magnesium sulfate refers to crystalline $MgSO_4 7H_2O$.

TABLE I*a*

The method of crystallization was essentially the same as that previously described (Northrop, 1930). The amorphous precipitate obtained with magnesium sulfate was washed with acid instead of dissolving in water and precipitating with acid. The amorphous precipitate must be filtered dry and the amount of acid used to wash must be about equal to the weight of the amorphous cake. If too little acid is used, too much magnesium sulfate is left in the precipitate and crystallization is uncertain. If too much acid is used the protein dissolves. The following directions apply only to crystallization of preparations which have been prepared from Cudahy pepsin by extraction with 0.6 saturated magnesium sulfate, 0.2 M pH 5.0 acetate. Such preparations crystallize much more easily and completely than preparations obtained by the original method when using Cudahy pepsin. The method of Philpot (1935) may also be used but the yield is much smaller.

Experimental Procedure

Suspension of amorphous pepsin in 0.8–0.9 saturated magnesium sulfate pH 2.5–3.0 filtered with suction on a large funnel so that the filter cake is less than 5 mm. thick. Filtration continued until precipitate begins to crack. Quantity of cold 0.02 N sulfuric acid equal to weight of the precipitate, approximately equivalent to 4 times the protein, poured on and filtered as dry as possible. Precipitate weighed and stirred with 1/4 its weight of water in tall, narrow beaker. Beaker placed in 1 liter jar of water at 35°C. and stirred into smooth paste. 0.5 M sodium hydroxide added very slowly with continuous stirring until the precipitate is just dissolved. pH should be 3.9–4.0. (If too much magnesium sulfate is left in the precipitate more sodium hydroxide is required. The pH of the clear solution is then 4.0–4.3.) The solution was stirred slowly, inoculated, although this is not essential, and allowed to cool slowly to 20°C. A heavy crystalline precipitate appeared and the suspension was allowed to stand at 20°C. for 10–20 hrs. The beaker was then placed in a large vacuum jar and the jar put in the ice box for 24 hrs. At the end of this time the crystallization was complete. If too much magnesium sulfate is present the crystals may be rounded or the suspension may set to a solid gel.

TABLE II

Preparation of Crystalline Pepsin, Sample No. 21, from Pepsinogen

Procedure the same as for preparation of solution No. 7 in Table I except that a different sample of pepsinogen was used.

Solution No. 7 precipitated by saturation with solid magnesium sulfate, filtered. Precipitate (92 gm.) extracted with 2300 ml. 0.6 saturated magnesium sulfate 0.2 M pH 5.0 acetate. Suspension filtered. Filtrate treated as described under solution No. 7 in Table I.

more soluble and the more insoluble components as would be predicted for a solid solution. The curve for the insoluble fraction also agrees with that expected for solid solutions rather than mixtures. In the case of mixtures the solid phase which first appears must be a pure or nearly pure component

but the curves show that this fraction gives again a smooth rounded curve, characteristic of a solid solution.

The preparation of several samples of the soluble fraction from various lots of Cudahy pepsin is shown in Tables III–VII. The solubility curves of these preparations in the amorphous form are shown in Fig. 2. The

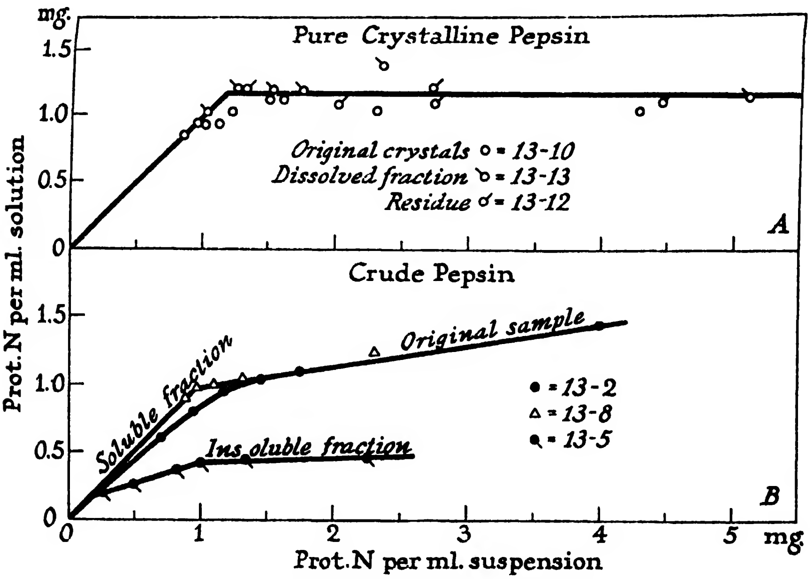

FIG. 1. Solubility curves of the amorphous form of various pepsin samples prepared from pepsinogen. Solvent, 0.50 saturated magnesium sulfate, 0.05 M pH 4.6 acetate buffer. In the method of plotting used in this and all subsequent solubility curves the theoretical curve for a system of one solid component has a slope of 1 up to the appearance of the solid phase and is parallel to the abscissa axis thereafter (slope 0) (Northrop and Kunitz, 1930; Kunitz and Northrop, 1938; Butler, 1940). In those figures where the experimental points lie on or close to this theoretical curve, no other curve has been drawn. In those figures where the experimental points lie far from the theoretical curve a separate curve has been drawn through the experimental point.

preparations obtained from the commercial preparation have the same activity and solubility as those from pepsinogen. The solubility (in the amorphous state) is constant in (0.5 saturated magnesium sulfate, 0.05 M pH 4.6 acetate) and also in (0.6 saturated magnesium sulfate, 0.2 M pH 5.0 acetate).

The absolute value for the solubility agrees with that previously reported (Northrop, 1930). Slight variations in this figure are due to slight variations in temperature, salt concentration, or non-protein nitrogen. The

primary interest is in comparison of the different values of the solubility in the same experiment and for this reason no special precaution was taken

TABLE III

Preparation of Crystals 22-29 from 1:10,000 U.S.P. Cudahy Pepsin

	No.	Quantity	N.P.N.	[P.U.]Hb/P.N.
		gm.	*per cent*	
500 gm. Cudahy pepsin dissolved in 1 liter 0.5 M pH 5.0 acetate buffer, 2 liters saturated magnesium sulfate solution and 200 gm. Filter-Cel added. Filtered. Precipitate washed 3 times with 500 ml. solvent and stirred 2 hrs. in 15 liters solvent. Filtered. Filtrate saturated with solid magnesium sulfate, filtered.				0.28
Precipitate....	1	200	20	0.32
Precipitate No. 1 stirred plus 200 ml. water, 0.5 M sodium hydroxide stirred in clear solution. 0.5 N sulfuric acid stirred in until pH was 2.5. Stood 6°C. 24 hrs., filtered, and crystallized. Precipitate....	19	40	10	0.34
40 gm. No. 19 plus 500 ml. (0.5 saturated magnesium sulfate, 0.1 M pH 4.0 acetate), stood 6°C. for 24 hrs. Filtered. Precipitate....	29	35	4	0.33

TABLE IV

Preparation of Sample 25-11

500 gm. Cudahy pepsin U.S.P. 1:10,000, lot No. 23, dissolved in 1 liter 0.5 M pH 5.0 acetate. 2 liters saturated magnesium sulfate and 200 gm. Filter-Cel added; filtered. Precipitate, washed with 1 liter solvent* and stirred with 15 liters solvent 2 hrs. at 20°C.; filtered. Filtrate: 3 kg. solid magnesium sulfate and 300 ml. 5 N sulfuric acid added and filtered. Precipitate– 80 gm. 80 gm. precipitate stirred with 40 ml. water, warmed to 37°C., and 0.5 N sodium hydroxide added till clear. Stirred and cooled slowly to 20°C. for 24 hrs. Small crop of large separate crystals (grey color), filtered. Precipitate, 25-6 (10 gm.). Filtrate: 100 ml. titrated to pH 3.0 with 0.2 N sulfuric acid, 100 ml. saturated magnesium sulfate added, filtered, and washed with 50 ml. cold N/50 sulfuric acid. 40 gm. precipitate crystallized (*cf.* Table I *a*) and filtered. Precipitate, 20 gm., 25-11.

* Solvent—0.6 saturated magnesium sulfate, 0.2 M pH 5.0 acetate buffer.

to have the composition of the solvent or the temperature *exactly* the same for different experiments.

The solubility is constant and independent of the amount of solid present. The preparation, therefore, is a pure protein, or a mixture of two proteins present in proportion to their solubilities or a solid solution of two or more

proteins having very nearly the same solubility (Northrop and Kunitz, 1930; Kunitz and Northrop, 1938; Butler, 1940).

It will be noted that crystallization does not change the solubility curves markedly and in the case of pepsin from pepsinogen improves them. The

TABLE V

Preparation of 0.35 Pepsin 28-6 and 28-28 from Cudahy 1:10,000 Pepsin

	No.	Quantity	N.P.N.	$\left[\frac{\text{P.U.}^{\text{Hb}}}{\text{P.N.}}\right]$
			per cent	
1 kg. Cudahy pepsin dissolved in 2 liters 0.5 M pH 5.0 acetate, 4 liters saturated magnesium sulfate and 300 gm. Filter-Cel added, filtered. Precipitate washed twice with 1 liter solvent. Precipitate stirred 20 hrs. with 4 liters solvent.				0.28
Filtered. Filtrate.......	1	4 liters		0.34
Precipitate....	2	1200 gm.		
Filtrate No. 1, saturated with solid magnesium sulfate, 60 gm. Filter-Cel added and filtered. Precipitate stirred with 300 ml. (0.2 saturated magnesium sulfate, 0.4 M pH 5.0 acetate) and filtered. 300 ml. saturated magnesium sulfate added to filtrate and filtered. Filtrate saturated with solid magnesium sulfate and filtered. Precipitate....	6	10 gm.	15	0.33
Precipitate No. 2, stirred with 6 liters (0.2 saturated magnesium sulfate, 0.4 M pH 4.0 acetate) filtered. Filtrate: 4 liters saturated magnesium sulfate added and filtered. Filtrate: Saturated with solid magnesium sulfate, 30 gm. Hyflo Super-Cel added, filtered, and washed with 0.6 saturated magnesium sulfate. Precipitate stirred with 2 liters (0.2 saturated magnesium sulfate, 0.4 pH 5.0 acetate), filtered. Filtrate: 2 liters saturated magnesium sulfate added, plus 30 gm. Hyflo Super-Cel and filtered. Filtrate: Saturated with solid magnesium sulfate plus 30 gm. Hyflo Super-Cel and filtered. Precipitate: 170 gm. stirred with 1 liter 0.2 saturated magnesium sulfate, 0.4 pH 5.0 acetate, filtered. Filtrate: 1 liter saturated magnesium sulfate added, very slight precipitate after 1 hr. Saturated with solid magnesium sulfate and filtered. Precipitate....	28	20 gm.	15	0.34

fact that the solubility is constant in two different solvents and is not changed by recrystallization, renders it very unlikely that the preparations consist of a mixture of proteins, present in proportion to their solubility or solid solutions of proteins having nearly the same solubility. This possibility, however, cannot be completely excluded but may be rendered more improbable by repeating the measurements in several other solvents.

TABLE VI

Preparation of 74-11, 74-56, and 74-58

	No.	Quantity	P.N./ml.
500 gm. Cudahy U.S.P. 1:10,000 pepsin (mixture of six different lots) dissolved in 1 liter 0.5 M pH 5.0 acetate buffer, 3 liters saturated magnesium sulfate, and 75 gm. Filter-Cel added. Filtered. Precipitate washed with 1 liter solvent.* Precipitate stirred 18 hrs. 20°C. in 14 liters solvent. Filtered. Filtrate.......	1	14 liters	0.35
5 kg. solid magnesium sulfate added to filtrate; filtered. Precipitate stirred with 3.5 liters solvent 18 hrs. 20°C. Suspension....	3	4 liters	1.4
Filtered. Filtrate.......	4		0.75
Filtrate: 800 gm. solid magnesium sulfate and 30 gm. Filter-Cel added and filtered. Precipitate: Stirred 6 hrs. 20°C. with 2 liters solvent. Suspension....	6	2.5 liters	1.2
Filtered. Filtrate.......	7		1.0
Filtrate 7: 400 gm. solid magnesium sulfate and 30 ml. 5 N sulfuric acid added, filtered, crystallized (*cf.* Table I *a*).......	11	50 gm.	
30 gm. No. 11 washed on filter with 100 ml. (0.5 saturated magnesium sulfate, 0.05 M pH 4.6 acetate) and stirred with 3 liters (0.5 saturated magnesium sulfate, 0.05 M pH 4.6 acetate) 20 hrs. 20°C. Filtered. Filtrate: 400 gm. solid magnesium sulfate, 50 ml. 5 N sulfuric acid added. Filtered and precipitate crystallized. Crystals......	30	15 gm.	
10 gm. 74-30 suspended in 1 liter (0.5 saturated magnesium sulfate, 0.1 M pH 4.0 acetate) 24 hrs. 6°C., filtered. Precipitate washed 3 times with (50 ml. 0.5 saturated magnesium sulfate, 0.05 M pH 4.6 acetate) and stirred with 500 ml. (0.5 saturated magnesium sulfate, 0.05 M pH 4.6 acetate) 20°C. 24 hrs.; filtered. Precipitate....	56	5 gm.	
Filtrate......	56F	500 ml.	
Precipitate 74-56 stirred with 50 ml. (0.5 saturated magnesium sulfate, 0.05 M pH 4.6 acetate) 24 hrs., 20°C., centrifuged. Supernatant...	561		0.18
Precipitate, above treatment repeated. Supernatant...	562		0.17
" " " " Supernatant...	563		0.17
Precipitate stirred with 15 ml. (0.5 saturated magnesium sulfate, 0.05 M pH 4.6 acetate) 24 hrs., 20°C., centrifuged. Supernatant...	564		0.20
Precipitate 74-56, above treatment repeated. Supernatant...	565		0.17
Precipitate....	566	0.5 gm.	
Filtrate 56F: 100 gm. solid magnesium sulfate, 10 ml. 5 N sulfuric acid added, filtered and washed, and precipitate crystallized. Crystals......	58	0.5 gm.	
58 suspended in 200 ml. (0.5 saturated magnesium sulfate, 0.1 M pH 4.0 acetate) 24 hrs., 6°C., filtered. Precipitate....	60	0.45 gm.	

* Solvent—0.6 saturated magnesium sulfate, 0.2 M pH 5.0 acetate buffer.

TABLE VII

Preparation of Sample 75-8

Mixed lot of six batches Cudahy pepsin U.S.P. 1:10,000. 1 kg. dissolved in 2 liters 0.5 M pH 5.0 acetate buffer, 4.5 liters saturated magnesium sulfate, and 50 gm. Hyflo Super-Cel added. Precipitate filtered and washed with 1 liter solvent.* Precipitate stirred 20 hrs. at 20°C. with 14 liters solvent, filtered, and washed with 1 liter solvent. 14 liters filtrate. 3 kg. solid magnesium sulfate added to filtrate and 100 gm. Filter-Cel and filtered. Precipitate washed with 1 liter solvent. Precipitate stirred with 2.5 liters solvent 20 hrs. at 20°C. Filtered. 500 gm. magnesium sulfate added to filtrate and 60 ml. 5 N sulfuric acid stirred in. Filtered and precipitate washed with 100 ml. cold N/50 sulfuric acid; precipitate, 100 gm. Crystallized. (*Cf.* Table I *a.*) 40 gm. good crystals, some in clusters. Crystalline precipitate filtered, washed with 200 ml. 0.5 saturated magnesium sulfate, 0.1 M pH 4.0 acetate. Suspended in 300 ml. 0.5 saturated magnesium sulfate, 0.1 M pH 4.0 acetate = 75-8.

* Solvent = 0.6 saturated magnesium sulfate, 0.2 M pH 5.0 acetate.

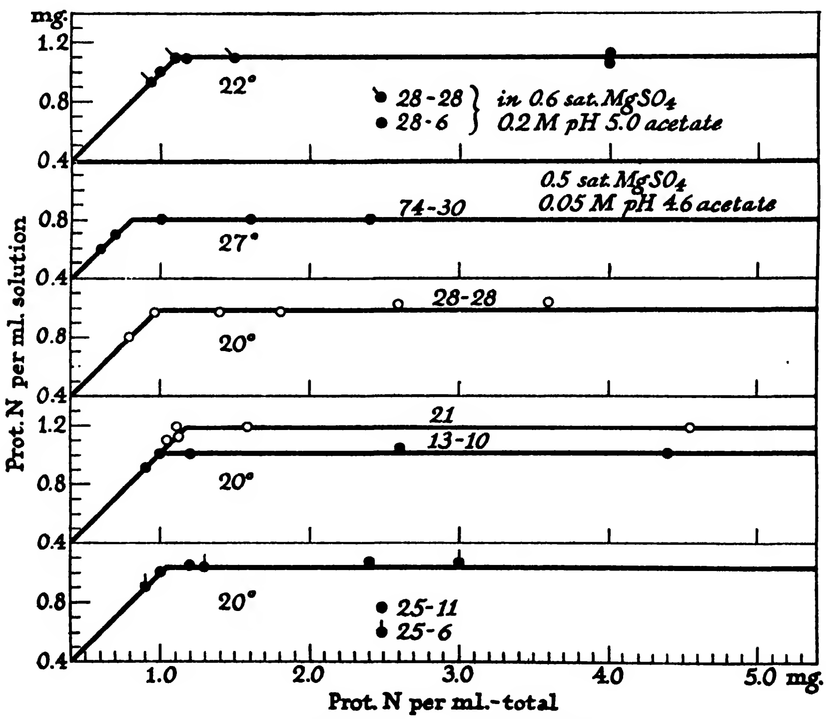

FIG. 2. Solubility curves of different samples of soluble pepsin in the amorphous form in magnesium sulfate solution.

From what is known of solubility in general and that of proteins in particular it is unlikely that two or more proteins would have even approximately the same solubility in a variety of solvents at different pH's.

In order to extend the solubility measurements to other solvents and to obtain values for the solubility of the crystalline protein the measurements were repeated with the crystalline protein in several different solvents.

Conditions Used in the Solubility Determinations.—In order to apply the phase rule to solubility curves the following conditions are necessary (Northrop and Kunitz, 1930; Butler, 1940; Kunitz and Northrop, 1938).

1. The system must be at equilibrium, *i.e.* the concentration of the various components must not change with time due either to a chemical reaction or to incomplete saturation of the solution with the solid.

TABLE VIII

Increase in Non-Protein Nitrogen in Various Solvents

Crystalline preparation 58-18 washed with (0.5 saturated magnesium sulfate, 0.05 M pH 4.6 acetate) and then with water; dissolved in water and 1 ml. solution added to 50 ml. of solvent noted. 15 ml. solution in test tube with glass beads and rotated twice per min. at 20°C. Analyzed for total and non-protein nitrogen at once and after 7 days.

Solvent	0.002 HCl 0.098 KCl		0.5 sat. $MgSO_4$ 0.05 pH 4.6 acetate		0.52 M Na_2SO_4 1×10^{-3} H_2SO_4		0.35 sat. $MgSO_4$ 1×10^{-3} H_2SO_4		0.9 sat. $MgSO_4$ 0.1 M Na $\bar{A}$	
Total N, *mg./ml.*	0.28		0.28		0.28		0.28		0.28	
Prot. N, *mg./ml.*	0.264		0.265		0.264		0.265			
N.P.N. at once, *mg./ml.*	0.015 0.017	0.016	0.016 0.017	0.0165	0.017 0.015	0.016	0.018 0.015	0.0165	(0.017)	
N.P.N. after 7 days, *mg./ml.*	0.080 0.078	0.079	0.044 0.042	0.043	0.028 0.033	0.030	0.035 0.034	0.0345	0.031 0.028	0.029
Increase in N.P.N., *mg./ml.*	0.063		0.0265		0.014		0.018		0.012	
Increase in N.P.N. as per cent of original value	400		160		90		108		90	
Protein decomposed, *per cent*	24		10		5.3		6.8		5	

2. The concentration of all components except the protein must be the same in all systems containing solid protein. If it is not, the system has one degree of freedom and no conclusion can be drawn concerning the nature or number of the protein components present.

In addition to these theoretical requirements it is necessary for practical reasons to choose a solvent in which the protein has an accurately measurable solubility.

The unstable nature of protein in general and pepsin in particular renders fulfillment of these requirements difficult. Pepsin is known to decompose in solution with the formation of non-protein products at a measurable rate under any conditions so far described.

The first solubility determination made by Northrop (1930) was done with M/100 hydrochloric acid. It was found that inactivation occurred so rapidly under these conditions as to render the experiment valueless. The rate of inactivation slowed down rapidly as the decomposition products collected in solution so that solutions which

already contained 10 per cent or more non-protein nitrogen appeared to be fairly stable. It is probably for this reason that Steinhardt considered the enzyme to be stable at pH 2.5. However, as soon as the non-protein nitrogen is removed more is rapidly formed.

The inactivation of solutions of pepsin in various solvents stirred gently at 20°C. (without any air bubbles) is shown in Table VIII. The table shows that in (0.098 potassium chloride, 0.002 hydrochloric acid) the non-protein nitrogen increases 400 per cent in 7 days equivalent to 24 per cent of the total protein present. In (0.5 saturated magnesium sulfate, 0.05 M pH 4.6 acetate) the corresponding figures are 160 per cent and 10 per cent, while in sodium sulfate, magnesium sulfate without acetate, or 0.9

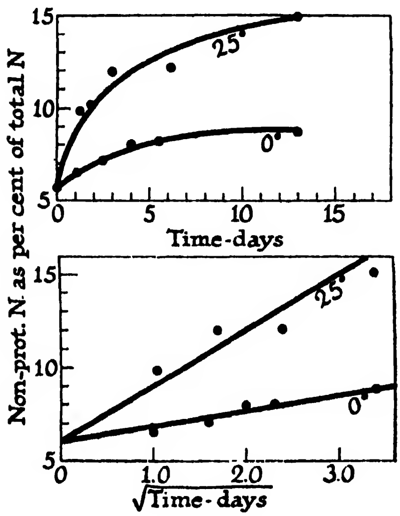

FIG. 3

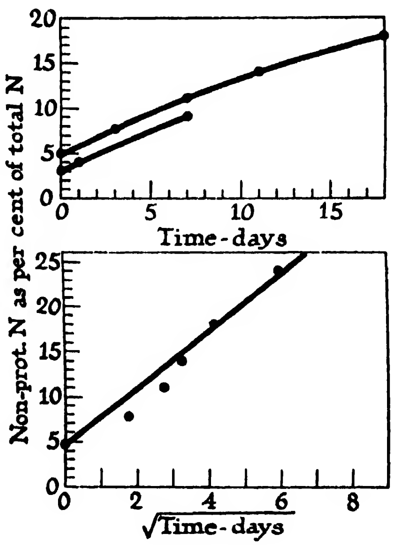

FIG. 4

FIG. 3. Decomposition of pepsin in 0.1 M pH 4.6 acetate buffer at 25°C. and 0°C.

FIG. 4. Decomposition of a suspension of crystalline pepsin in 0.1 M potassium chloride pH 2.7 and 5°C.

saturated magnesium sulfate, 0.1 M sodium acetate (pH 6.0) they are about 100 per cent and 5–7 per cent. It requires 1–3 days for equilibrium to be reached under these conditions so that even in the most favorable solvent there is significant decomposition while in dilute hydrochloric acid, decomposition is so marked as to render this solvent unsuitable.

Fig. 3 shows the rate of decomposition of pepsin in solution in N/10 pH 4.6 acetate buffer at 25°C. and 0°C. It will be noted that the rate of inactivation apparently decreases rapidly after reaching 9 per cent at 0°C. and 15 per cent at 25°C. However, if the results are plotted against the square root of the time it is evident that the rate is nearly constant and is proportional to the square root of the time. The square root relation is characteristic of pepsin hydrolysis curves and is probably the result of the inhibiting action of the products of the reaction. The decomposition of a suspension of crystalline pepsin in 0.1 M potassium chloride pH 2.7 at 5°C. without stirring is shown in

Fig. 4. Even under these conditions the inactivation is much too rapid to allow the use of the solvent. Comparison of these results with those in Table VIII shows that the per cent increase in non-protein nitrogen in the suspension is less than that in the solution at nearly the same pH. The increase in non-protein nitrogen expressed as per cent of the dissolved protein is, however, nearly the same. Decomposition occurs in solution therefore and not in the solid protein, as is to be expected. For this reason the formation of non-protein nitrogen in a *concentrated* suspension may be too small to be detected.

TABLE IX

A. Formation of Non-Protein Nitrogen under Different Conditions

In 0.1 M pH 4.6 Acetate Buffer

5 gm. crystalline pepsin in 35 ml. M/10 pH 4.6 acetate. Dialyzed at 7°C. against M/10 pH 4.65 acetate buffer.

Time dialyzed	Per cent N.P.N. inside	Total N.P.N.	Total N.P.N. in dialysate
days		*mg.*	*mg.*
0	12	30	0
2			26
5			37
7	1.5		41

B. In 0.4 Saturated Magnesium Sulfate and 0.5 M pH 4.6 Acetate

5 gm. crystalline pepsin stirred for 20 min. at 22°C. with 20 ml. solvent. Centrifuged and supernatant analyzed for protein and non-protein nitrogen. Repeated 6 times.

	Protein nitrogen	Non-protein nitrogen
	mg.	*mg.*
Original suspension	221.5	8.5
In combined extracts	189.8	17.7
In residue	23	1.15
Total present after extractions	212.8	18.85
Protein nitrogen lost	8.7	
Non-protein nitrogen formed		10.3

C. Solution of Pepsin 6-17, 0.33 $[P.U.]^{Hb}_{P.N.}$ in Water Adjusted to Various pH with Hydrochloric Acid

Temperature 0°C. 1 mg. nitrogen/ml.

pH	4.6	3.5	2.5
	Non-protein nitrogen as per cent of total nitrogen		
After 0 hrs.	3	3	3
" 70 "	7	12	12
Per cent increase non-protein nitrogen	125	300	300

The formation of non-protein nitrogen during dialysis against M/10 pH 4.6 acetate or during repeated washing with (0.5 M saturated magnesium sulfate, 0.05 M pH 4.6 acetate) is shown in Table IX. In both cases the amount of non-protein nitrogen eventually formed in solution is considerably greater than the total amount originally present show-

ing that it is actually formed during the experiment. This is confirmed in Table IX *B* since the loss of protein nitrogen is equal, within the experimental error, to the gain in non-protein nitrogen.

In Table IX *C* the formation of non-protein nitrogen in solution at 0°C. and pH 4.6, 3.5, and 2.5 is shown. This sample was unusually low in non-protein nitrogen at the beginning of the reaction and the rate of formation is correspondingly higher. It is evident from the foregoing that there is no general relation between the per cent of non-protein nitrogen in the solid and in the solvent but that the ratio varies depending on the solvent used. In 0.5 saturated magnesium sulfate and 0.1 M pH 4.0 acetate, for instance, only non-protein nitrogen appears in solution and no protein nitrogen. Nevertheless, it is not possible to completely remove the non-protein nitrogen in any solvent so far used since the diffusion of the non-protein compounds into the solution is slow and in the meantime more non-protein nitrogen is formed.

It is evident from these results that pepsin decomposes to a measurable extent in all solutes tried, in the course of several days. Solubility determinations must therefore be carried out as rapidly as possible. The solubility of the amorphous form may be determined in less than 1 hour and in this time no detectable decomposition will occur. The method has the further advantage that no stirring is necessary. The method has the serious disadvantage, however, that there is always some uncertainty as to whether the values obtained are equilibrium values or not.

The amorphous form is unstable and at true equilibrium must all change over to the crystalline state. It follows necessarily that the amorphous form is more soluble. It is possible, however, by working rapidly under conditions where the protein does not crystallize easily to obtain values for the solubility of the amorphous form which are equilibrium values in the sense that the same value is reached by precipitating or by dissolving the solid. The measurements are made by diluting a suspension of the crystals or amorphous protein in the solvent to be used, with a part of the solvent in which the protein is soluble and then adding the other part of the solvent so that the final solutions all have the same pH and salt concentration. In the case of (0.5 saturated magnesium sulfate, 0.05 M pH 4.6 acetate) the protein is washed several times with the solvent and the precipitate then dissolved in 0.1 M pH 4.6 acetate. If the protein is in crystalline form it is essential to allow the solution to stand several hours so as to be sure all crystals are dissolved, otherwise crystallization will occur as soon as the salt solution is added. Pepsin does not crystallize readily in this solvent unless crystals are already present to serve as inoculation (*cf.* Northrop, 1930). An amount of saturated magnesium sulfate exactly equal to the quantity of acetate previously added is then added slowly with constant stirring so as to avoid local excess and subsequent precipitation. The tube is allowed to stand for 1–4 hours and then filtered. If filtered too soon the solution may be supersaturated and this is apt to be the case when the amount of precipitate is very small. If allowed to stand too long crystallization will start. It has been found that the value after 1 hour standing is fairly constant and agrees quite closely with that obtained by stirring the solid with the solvent. An example of an experiment which shows the effect of time of standing and of the presence of inoculating crystals is shown in Table X.

Solubility of the Crystalline Protein. Time for Equilibrium to Be Reached.—It is evident from the inactivation experiments that some decomposition occurs even in 24 hours. It is necessary therefore to obtain solubility equilibrium as rapidly as possible. The

rate of solution, other things being constant, is proportional to the rate of stirring but unfortunately the rate of decomposition also increases with the rate of stirring and, if foaming occurs, may become very great. The method of rapid stirring described earlier (Northrop, 1930) gives very quick saturation but it is difficult to obtain stirrers sufficiently free from vibration. It was found that rotating the suspension in 10 × 0.7 cm. tubes containing 3–5 glass beads 2 mm. in diameter gave nearly constant solubility values in from 6–24 hours. The tubes are rotated around their short axis about 5 times per minute. Under these conditions the concentration of protein nitrogen in sodium

TABLE X

Effect of Time of Standing at 20°C. on Solubility of Amorphous Pepsin in {0.5 Saturated Magnesium Sulfate, 0.05 M pH 4.6 Acetate}

Crystals 74-30 washed with (0.5 saturated magnesium sulfate, 0.05 M pH 4.6 acetate) and suspended in same solvent. Suspension I. 5 mg. N/ml.

	A 5 ml. suspension I + 10 ml. 0.10 M pH 4.6 acetate, stood until nearly clear and 10 ml. saturated magnesium sulfate added, 20°C. Stood at 20°C. and samples filtered and analyzed for protein nitrogen as noted. Total P.N. = 1.0 mg./ml.	*B* Same as A but stood in acetate 4 hrs. before addition of saturated magnesium sulfate. Total P.N. = 1.0 mg./ml.
Time after addition of saturated magnesium sulfate		
	P.N./ml. filtrate	*P.N./ml. filtrate*
1 min.	1.0	1.0
10 min.	0.90	1.0
1 hr.	0.70	0.90
6 hrs.	0.50 (highly refractile large particles)	0.90 fine amorphous precipitate

Suspension of amorphous precipitate added slowly to 10 ml. solvent until slight permanent turbidity. Total nitrogen/ml. = 1.0. Nitrogen/ml. dissolved = 0.85.

sulfate solutions remains constant within 3 per cent from 6 to 24 hours at 20°C. There is a slow steady increase over longer periods of time. Similar results were obtained in the other solvents except that in some experiments the protein concentration in (0.5 saturated magnesium sulfate, 0.05 M pH 4.6 acetate) increased 10–20 per cent per day. After 48–72 hours a secondary more rapid increase frequently occurs which is probably due to the growth of mold in the suspensions.

All experiments were done by stirring the precipitate with several successive portions of the solvent until a constant value for the protein nitrogen per milliliter was obtained on two or more successive solutions. Equilibrium is obtained in a few hours in such concentrated suspensions and the per cent of the total protein dissolved is small. Variation in these preliminary washings must therefore be due to changes in the concentration of

one or more components of the solvent (*cf.* Butler, 1940). Conversely, if the solubility of the protein is constant in successive washings it may be assumed that the composition of the solvent is constant. The suspension is then diluted with the solvent so as to obtain a series of suspensions containing varying quantities of solid protein and the tubes are rotated for 6–24 hours at 20°C. They are then filtered through Whatman No. 42 paper and the filtrate analyzed. This method of distribution is not so sensitive as the method of successive washings. The latter method, however, necessarily extends over many days and is evidently inapplicable to such an unstable protein as pepsin.

Non-Protein Nitrogen.—The experiments on the rate of decomposition of pepsin show that significant quantities of non-protein nitrogen are formed even in 24 hours at 20°C. so that these compounds must be considered as components of the system. In this case there are five components, water, salt, acid, non-protein nitrogen,[1] and protein, and two phases, solid and solution. If the temperature, pressure, acid, non-protein nitrogen,

TABLE XI

Per Cent Non-Protein Nitrogen in Precipitate from Dilute Suspension and in Supernatant from Concentrated Suspension in Various Solvents

Solvent	Precipitate	Non-protein nitrogen as per cent of total nitrogen	
		In precipitate	In supernatant
(0.5 saturated magnesium sulfate, 0.05 M pH 4.6 acetate)	Amorphous	5.0	5.0
	Crystals, average of 13 analyses	5.1	15.3
0.52 M sodium sulfate	Crystals	4.8	9.0
(0.9 saturated magnesium sulfate, 0.1 M sodium acetate)	Crystals	6.0	10.0

and salt are constant, and only one protein component is present, the system is fixed and the protein concentration must be constant. If the protein is not constant under these conditions then there must be more than one protein component.

If, however, the non-protein concentration varies in the system then the system will have one degree of freedom even though the salt and acid are constant. In this case variation in the protein concentration does not necessarily prove the presence of more than one protein component.

Before it is possible to interpret the solubility curves, therefore, it is necessary to know whether or not the non-protein nitrogen is constant. In order to do this the precipitate from a dilute suspension and the filtrate from a concentrated suspension in the various solvents under consideration were analyzed. Any variation in composition would be expected to appear in these two fractions, whether the system is considered a mixture or solid solution. If the composition of these fractions is the same the composition of all the others may be considered constant. Table XI shows that with (0.5

[1] Actually there are undoubtedly a number of different non-protein components present, but since no method of analysis is available for the individual components they are considered as one.

saturated magnesium sulfate, 0.05 M pH 4.6 acetate) the per cent non-protein nitrogen in the *amorphous* precipitate is the same as that in the filtrate. Any variation in the dissolved protein concentration in this system therefore indicates the presence of more than one protein component while constant protein concentration indicates the presence of only one protein component. In the case of the crystals, however, this is not the case. The per cent of non-protein nitrogen is higher in the filtrate than in the precipitate. In this case, therefore, there is still one degree of freedom, even though the acid and salt are fixed and variations in protein concentration do not necessarily indicate the presence of more than one protein component.

The results of the solubility measurements on various preparations of the crystalline protein are shown in Figs. 5-8.

The curves show that the solubility is constant and independent of the amount of solid phase present in the following solvents. Amorphous form in (0.5 saturated magnesium sulfate, 0.05 M pH 4.6 acetate) or (0.6 saturated magnesium sulfate, 0.2 M pH 5.0 acetate). Crystalline form in 0.76 M sodium sulfate pH 4.1, 0.52 M sodium sulfate pH 3.9, 0.9 saturated magnesium sulfate pH 6.0, 0.1 M sodium acetate, 3.2 M sodium chloride pH 3.8, or 0.4 saturated magnesium sulfate pH 3.8.

The solubility of both the amorphous and crystalline forms in (0.5 saturated magnesium sulfate, 0.05 M pH 4.6 acetate) and of the crystalline form in 0.52 M sodium sulfate agrees closely with that previously determined (Northrop, 1930).

The original and the present preparations both gave constant solubility in the amorphous form in (0.5 saturated magnesium sulfate, 0.05 M pH 4.6 acetate) and in the crystalline form in sodium sulfate. The solubility curves of the crystals in (0.5 saturated magnesium sulfate, 0.05 M pH 4.6 acetate) are not constant but are rounded in the dilute suspensions. This abnormality is discussed below.

The earlier experiments were made with pepsin which had been crystallized directly from Parke, Davis preparation without previous fractionation of the protein. Pepsin prepared in this way from commercial preparations now available does not give constant solubility in any solvent tried. The original preparation was repeated many times but only one lot of commercial pepsin was used. Evidently this particular lot of crude pepsin was more homogeneous than usual.

In (0.2 M pH 4.4 acetate, 1.0 M sodium sulfate), (0.2 M pH 3.5 acetate, 0.5 M sodium sulfate) or (0.5 saturated magnesium sulfate, 0.05 M pH 4.6 acetate) however, the points do not fall on the theoretical curve for a system of one solid phase of one component but are below this curve in the dilute suspensions (Fig. 5). The same result was obtained in the original solubil-

ity experiments. All these solvents contain acetate buffer, while the corresponding solvents without the acetate buffer give theoretical curves.

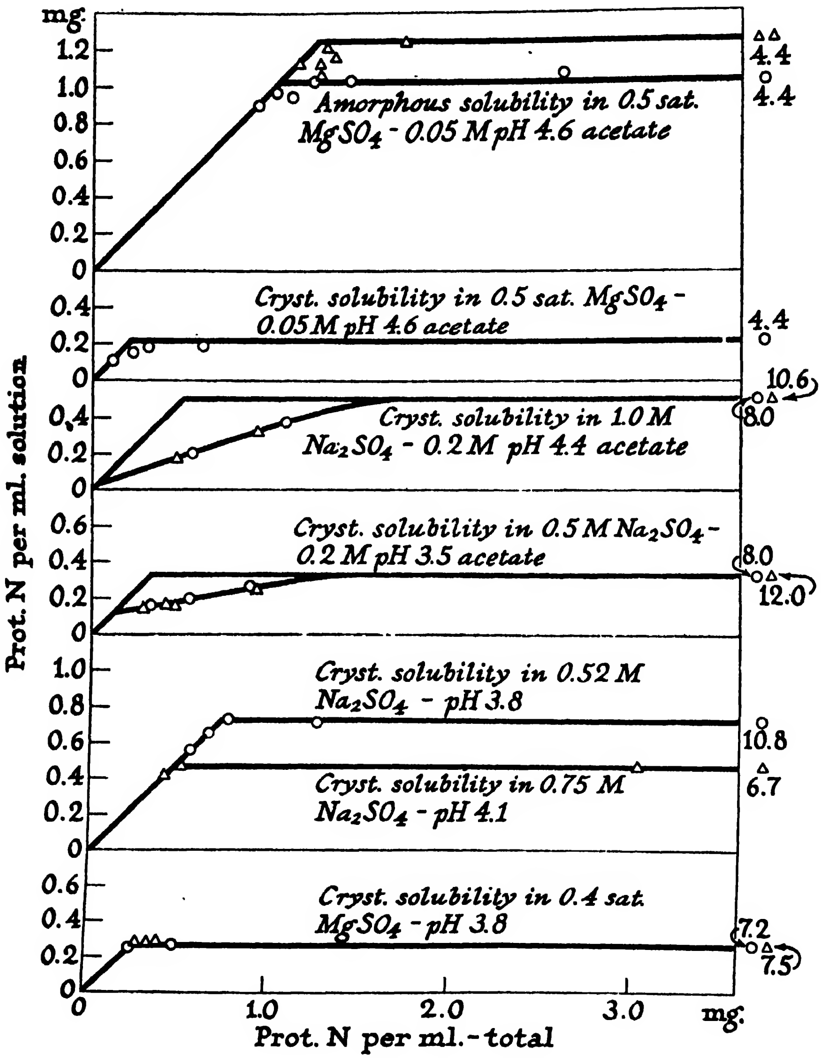

FIG. 5. Solubility of crystalline pepsin, sample No. 13-10 circles and No. 21 triangles, in various solvents at 20°C. For preparation of these samples see Tables I and II.

There are two possible explanations for this discrepancy:

1. The variation in non-protein nitrogen affects the solubility of the protein in the presence of acetate buffer but does not do so in the other solvents. This explanation predicts that the addition of non-protein nitro-

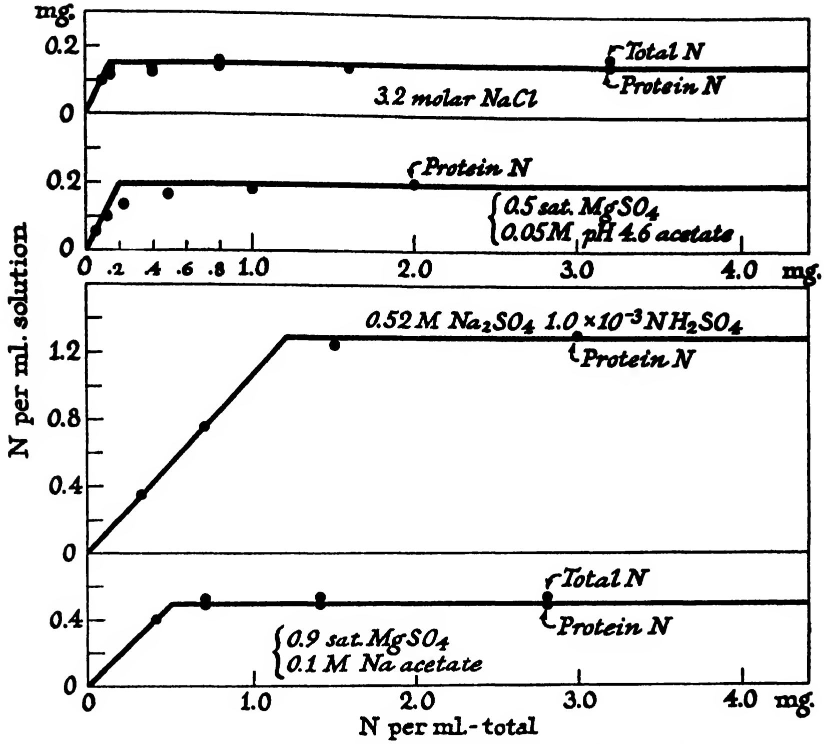

FIG. 6. Solubility of crystalline preparation No. 75-8 (see Table VII) in various solvents at 20°C.

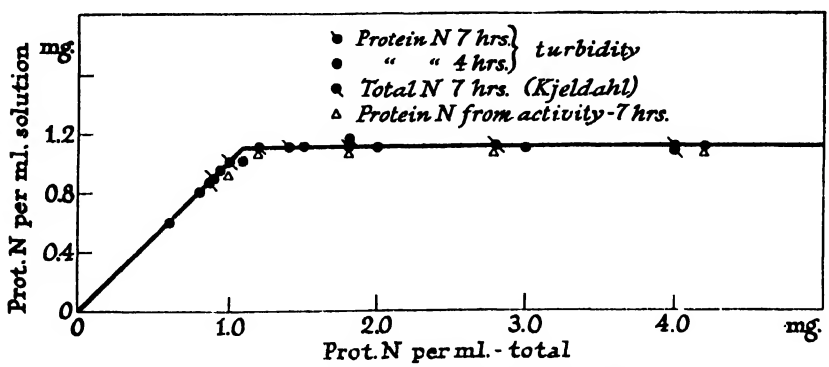

FIG. 7. Solubility of crystalline preparation No. 22-29 (see Table III) in 0.52 M sodium sulfate, 2.5 × 10^{-3} sulfuric acid at 20°C.

gen will affect the solubility of the protein in the presence of acetate but will not do so in the absence of acetate buffer. As will be seen below this is the case.

2. More than one protein is present. The solubilities of these proteins are nearly the same in the absence of acetate but are different in the presence of acetate. This explanation predicts that the protein can be further separated by fractionation in the solvents containing acetate. The results show that this cannot be done.

Effect of Non-Protein Nitrogen on the Solubility of Pepsin in (0.5 Saturated Magnesium Sulfate, 0.05 M pH 6.4 Acetate) and in 0.52 M Sodium Sulfate, pH 3.9.—The substances grouped together as non-protein nitrogen may be quite different in different solvents. All that is known about them is that they are formed by decomposition of the protein and that they do not precipitate with 2 per cent trichloracetic acid. In order to test their effect on the solubility of the protein therefore it is necessary to have a solution prepared

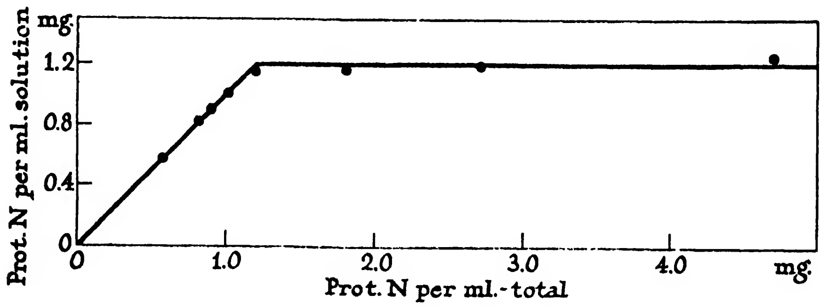

FIG. 8. Solubility of crystalline preparation No. 25-11 (see Table IV) in 0.52 M sodium sulfate, 2.5×10^{-3} sulfuric acid at 20°C. Protein nitrogen determined by turbidity.

as nearly as possible under the actual conditions of the solubility experiment. The solution containing the non-protein nitrogen was prepared therefore from a saturated solution of the protein in the solvent by heating the solution as rapidly as possible and filtering off the denatured protein. The details of this procedure are shown in Table XII. There is no change in the pH or non-protein nitrogen content of the magnesium sulfate-acetate solvent upon removal of the protein in this way. In sodium sulfate the non-protein nitrogen increases and the pH becomes slightly more alkaline upon heating. This difference is due to the fact that pepsin is more active in the less concentrated sodium sulfate than it is in the magnesium sulfate so that more non-protein nitrogen is formed from the denatured protein before inactivation is complete.

The effect of the solvents on the solubility of the protein is shown in Fig. 9. The solubility in the magnesium sulfate-acetate solvent is decreased markedly by the presence of the non-protein nitrogen and the resultant

curve deviates more from the theoretical than does the curve in which pure solvent was used. It must be remembered that the suspension in the pure solvent also contains the non-protein nitrogen which is formed during the solubility experiment. It is evident that this effect of non-protein nitrogen on the solubility is quite sufficient to account for the deviation of the results from the theoretical curve in this solvent. It is quite possible that this effect of the non-protein nitrogen is due to the formation of a solid

TABLE XII

Preparation of "Non-Protein Nitrogen Solvents" Fig. 9

	No.	Nitrogen/ml.		pH
		Protein	Non-protein	
(0.5 saturated magnesium sulfate, 0.05 M pH 4.6 acetate)				
Suspension of sample 54-19 in (0.5 saturated magnesium sulfate, 0.05 M pH 4.6 acetate)	1	0.50	0	3.90
Suspension No. 1 rocked 20°C., 24 hrs.	2	0.50	0.03	3.90
Filtered. Filtrate	3	0.20	0.03	3.90
Filtrate No. 3, heated to 100°C. for 1 min., cooled, and filtered	4	0	0.05	3.90
Solution No. 4 used in solubility experiment.				
Non-protein nitrogen in sodium sulfate solvent.				
Suspension of 54-19 in 0.52 M sodium sulfate, 1×10^{-3} M sulfuric acid	5	1.6	0	3.90
Suspension No. 5 rocked 20°C., 24 hrs.	6	1.5	0.08	3.90
Suspension No. 6 filtered. Filtrate	7	0.65	0.08	3.90
Filtrate No. 7 heated rapidly to 100°C. for 1 min., cooled, and filtered	8	0	0.45	3.98
0.2 ml. 0.2 M hydrochloric acid added to 50 ml. solution No. 8	9	0	0.45	3.90
Solution No. 9 used in solubility experiment.				

solution of the non-protein with the protein as Steinhardt has suggested (1938, 1939). Experimental test of this mechanism is difficult since the amount of non-protein nitrogen in the solid is too small to determine accurately. Expressed as mole fraction, however, it may be very large owing to the great difference in molecular weight between the protein and non-protein components.

In sodium sulfate the non-protein nitrogen has less effect on the solubility of the protein and what effect it does have is in the opposite direction, that is, the protein is slightly more soluble in the presence of increased amounts of non-protein nitrogen. In this case the non-protein nitrogen added was

4 times that formed during the solubility experiment. Evidently the amount of non-protein nitrogen formed during the experiment would not affect the solubility to a measurable extent. The non-protein nitrogen may therefore be considered an inert component in this solvent.

Results of Fractionation in 0.5 Saturated Magnesium Sulfate, pH 4.6 Acetate.—The preceding section shows that the effect of non-protein nitrogen on the solubility of the protein is sufficient to account for the divergence of the results from the curve for a pure protein. This result, however, does

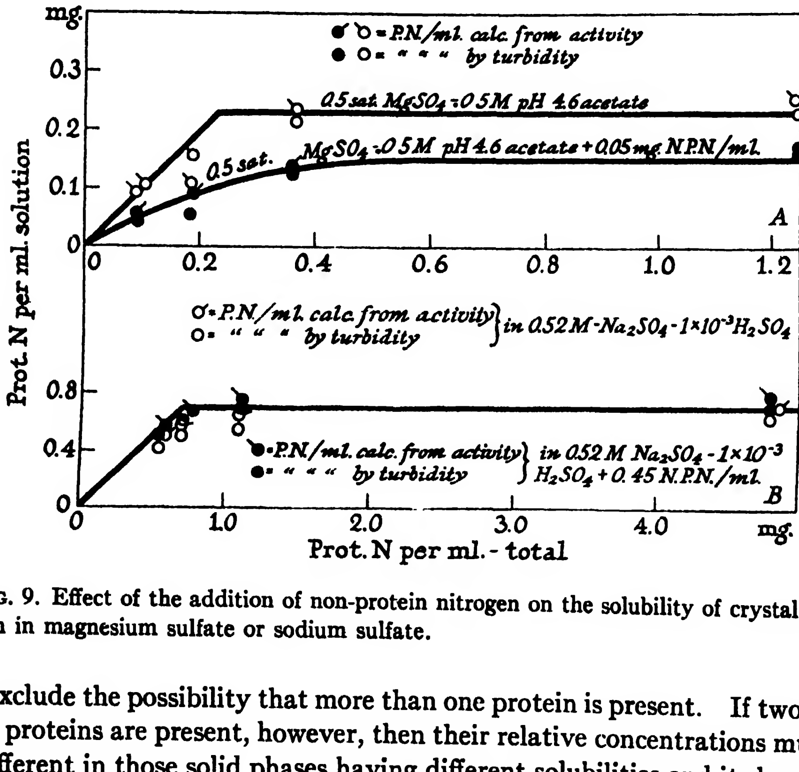

FIG. 9. Effect of the addition of non-protein nitrogen on the solubility of crystalline pepsin in magnesium sulfate or sodium sulfate.

not exclude the possibility that more than one protein is present. If two or more proteins are present, however, then their relative concentrations must be different in those solid phases having different solubilities and it should, therefore, be possible to separate them at least partially by reextracting the protein with the solvent. The extract obtained when only a small per cent of the solid is dissolved should contain more of the soluble component while the residue left after most of the protein is dissolved should be high in the insoluble component. It may be recalled that such a separation does occur with crude preparations which give solid solution curves in all solvents (Fig. 1). Preparations giving good curves in other solvents but solid solution curves in (0.5 saturated magnesium sulfate, 0.05 M pH 4.6 acetate)

cannot be separated by extraction with this solvent. The protein extracted gives curves differing slightly if at all from the residue left after nearly complete solution. Both curves are indistinguishable from that of the original preparation (Fig. 10). The figure shows that preparation 74-11 gave a solid solution curve when tested in the amorphous form. Preparation 74-30 was prepared from 74-11 by extraction in the solvent used for solubility measurements. This preparation (74-30) has constant solubility in the amorphous form. This preparation, however, gives a slightly rounded curve in the crystalline form in (0.5 saturated magnesium sulfate,

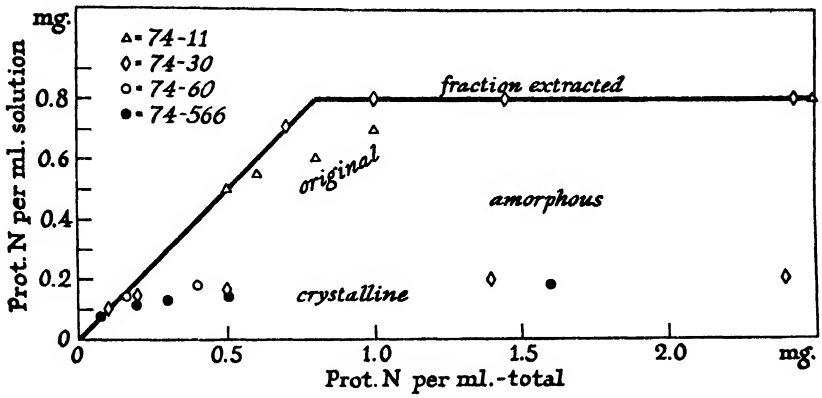

FIG. 10. Effect of fractionation with 0.5 saturated magnesium sulfate, 0.05 M pH 4.6 acetate on the solubility of pepsin preparations. Solubility determined in 0.5 saturated magnesium sulfate, 0.05 M pH 4.6 acetate. 74-11, original crystals; 74-30, 50 per cent of 74-11 extracted with the solvent, precipitated, and crystallized; 74-60, 10 per cent of 74-30 extracted in the solvent, precipitated, and crystallized; 74-566, residue left after repeated extraction of 74-30 in the solvent, equivalent to 5 per cent of 74-30.

0.05 M pH 4.6 acetate). Upon further extraction the same curve is obtained either from the residue or from the protein extracted (lower curves in Fig. 9).

It may be concluded, therefore, that the preparation contains only one protein component and that the rounded curves obtained in (0.5 saturated magnesium sulfate, 0.05 M pH 4.6 acetate) are due to variation in the non-protein nitrogen.

II. Preparation of a Less Soluble Fraction

The preliminary experiments reported by Desreux and Herriott (1939) indicate the presence of at least one other less soluble component, besides the one described in the preceding section. This insoluble component is present in large amounts in Parke, Davis pepsin. Accordingly attempts to

TABLE XIII

Preparation of Insoluble Pepsin Sample 33-39

Precipitation between 0.1–0.45 and 0.45–0.6 saturated magnesium sulfate at pH 5.0.

	No.	Per cent sat. $MgSO_4$	Quantity	N.P.N.	[P.U.]Hb / [P.N.]
			gm.	*per cent*	
1 kg. Parke, Davis U.S.P. 1:10,000 pepsin dissolved in 5 liters 0.2 M pH 5.0 acetate, 3 kg. solid magnesium sulfate added and 300 gm. Hyflo Super-Cel and filtered. Filtrate.......	1	0.65			0.25
Precipitate washed with 500 ml. (0.6 saturated magnesium sulfate 0.2 M pH 5.0 acetate). Stirred with 5 liters (0.45 saturated magnesium sulfate, 0.2 M pH 5.0 acetate), filtered. Filtrate.......	2	0.45			
Precipitate....	3		35		0.20
Filtrate 2: 900 gm. solid magnesium sulfate added, titrated to pH 5.0, filtered. Precipitate....	5	0.65	280		0.23
Precipitate 5 stirred in 1 liter (0.45 saturated magnesium sulfate, 0.5 M pH 5.0 acetate) and filtered..............		0.60			
400 ml. saturated magnesium sulfate added to filtrate and filtered. Precipitate....	8		140		
140 gm. precipitate 8 stirred with 900 ml. (0.45 saturated magnesium sulfate, 0.5 M pH 5.0 acetate), nearly clear, filtered..		0.45			
370 ml. saturated magnesium sulfate added to filtrate and filtered. Precipitate (120 gm.) stirred with 600 ml. (0.45 saturated magnesium sulfate, 0.5 M pH 5.0 acetate) and filtered. 240 ml. saturated magnesium sulfate added to 700 ml. filtrate and filtered. Precipitate (70 gm.) stirred with 350 ml. (0.45 saturated magnesium sulfate, 0.1 M pH 5.0 acetate) and filtered. 140 ml. saturated magnesium sulfate added to filtrate. Stood 24 hrs. 20°C. and filtered. Precipitate (40 gm.) stirred with 400 ml. 0.5 M pH 5.0 acetate, 600 ml. saturated magnesium sulfate added and filtered. Filtrate.......	19				0.25
Precipitate....	20		40		0.20

Preparation of Fraction Insoluble in 0.45 Saturated Magnesium Sulfate

Precipitate 33-3 (35 gm.) dissolved in 1 liter 0.5 M pH 5.0 acetate, 800 ml. saturated magnesium sulfate added, filtered. Precipitate....	32	0.45	20		
20 gm. precipitate 32 dissolved in 100 ml. 0.5 M pH 5.0 acetate, 80 ml. saturated magnesium sulfate added, filtered. Filtrate.......	33	0.45			0.33
Precipitate....	34		16		0.21

TABLE XIII—*Concluded*

	No.	Per cent sat. $MgSO_4$	Quantity	N.P.N.	$[P.U.^{Hb}_{P.N.}]$
			gm.	per cent	
16 gm. precipitate 34 dissolved in 50 ml. 0.1 M pH 4.6 acetate and 50 ml. saturated magnesium sulfate added. Filtered. Precipitate washed with (0.5 saturated magnesium sulfate, 0.05 M pH 5.0 acetate) and dissolved in 50 ml. 0.10 M pH 4.6 acetate. 50 ml. saturated magnesium sulfate and 30 ml. 5 N sulfuric acid added and filtered. Precipitate....	39		10		
8 gm. precipitate 39 dissolved in 60 ml. water, cooled to 0°C., and 60 ml. 5 N sulfuric acid and 1 gm. Filter-Cel added and filtered. Precipitate dissolved in 40 ml. 2 M pH 5.0 acetate, 40 ml. saturated magnesium sulfate added and filtered. Precipitate....	40			30	0.17
Precipitate 40 washed with (0.5 saturated magnesium sulfate, 0.05 M pH 4.6 acetate) dissolved in 55 ml. 0.1 M pH 4.6 acetate and 20 ml. saturated magnesium sulfate added; filtered. Precipitate....	43			50	0.12
Filtrate.......	42				
Filtrate No. 42: 10 ml. saturated magnesium sulfate added and filtered. Precipitate....	45			40	0.16
Filtrate.......	44				
Filtrate No. 44: 20 ml. saturated magnesium sulfate added and filtered. Precipitate....	47			30	0.20
Filtrate.......	46				
Filtrate No. 46 saturated with solid magnesium sulfate and filtered. Precipitate....	48			10	0.24

prepare the component were made with Parke, Davis 1:10,000 pepsin. The fractionation was carried out as described for the soluble component except that in this case the fraction precipitating between 0.10 0.45 saturated magnesium sulfate in 0.2 M pH 5.0 acetate was concentrated. Attempts were also made to isolate a fraction soluble between 0.45 and 0.60 saturated magnesium sulfate. The results of such a fractionation are reported in experiment 33, Table XIII. It is evident that the fraction soluble in 0.45 saturated magnesium sulfate and insoluble in 0.65 saturated magnesium sulfate decreases rapidly at each successive precipitation and that there is no indication that any constant value will be reached. This result does not exclude the existence of such a fraction but merely shows that, if present, isolation would entail the use of very large quantities of material. The fraction insoluble in 0.4 saturated magnesium sulfate also decreases rapidly but at each precipitation the substance becomes more

insoluble and a preparation is finally obtained which has a very low solubility in 0.45 saturated magnesium sulfate 0.2 M pH 5.0 acetate. The specific activity by the hemoglobin method is about 0.2. However, the fraction dissolved in the solvent has an activity of 0.3 so that the preparation is evi-

TABLE XIV

Preparation of Insoluble Pepsin 46-35 from Parke, Davis 1:10,000 Pepsin by Repeated Precipitation with 0.5 Saturated Magnesium Sulfate in pH 4.6 Acetate

	No.	Quantity	Non-protein nitrogen	$\frac{P.U.}{P.N.}$
			per cent	
1 kg. Parke, Davis pepsin U.S.P. 1:10,000 in 2 liters 0.5 M pH 4.6 acetate. 100 gm. Filter-Cel added and filtered. Filtrate.......	1	2.5 liters		0.27
2.5 liters saturated magnesium sulfate and 100 gm. Filter-Cel added to 2.5 liters filtrate No. 1 and filtered. Precipitate....	3			
Precipitate No. 3 dissolved in 1 liter 0.5 M pH 4.6 acetate, 1 liter saturated magnesium sulfate added and filtered. Precipitate				
Solution and precipitation repeated 4 times. Precipitate....	13		18	0.22
Solution and precipitation repeated 12 times more. Precipitate....	33	60 gm.	25	0.20
Precipitate No. 33 dissolved in 200 ml. 0.10 M pH 4.6 acetate and 200 ml. saturated magnesium sulfate added. Suspension....	35			0.20
Crystallization				
10 gm. precipitate No. 33 washed with 5 ml. N/50 sulfuric acid, crystallized..................................	47-1	4 gm.		0.18
Precipitation at pH 4.0				
10 gm. 46-33 dissolved in 100 ml. water, dialyzed against water 24 hrs. Titrated to pH 4.0. Stood at 6°C. for 24 hrs., heavy precipitate, filtered. Filtrate.......			10	0.20
Precipitate dissolved in 100 ml. water, titrated to pH 3.3, centrifuged. Precipitate....	46A4			0.19

dently not homogeneous. Further fractionation at pH 4.65 with varying concentrations of magnesium sulfate (No. 43–48) yielded a series of preparations of increasing activity and solubility. It cannot be decided from these results whether the preparation consists of an inactive and insoluble protein with a single active protein or whether there are two or more active proteins. The fractionation at pH 4.6 is sharper than at pH 5.0 and a second sample of the insoluble protein was therefore prepared by repeated precipitation with 0.5 saturated magnesium sulfate in 0.25 pH 4.6 acetate (Table XIV).

Comparison of Specific Activity and Inactivation Rate of the Soluble and Insoluble Fraction.—If two active proteins are present it would be expected that the relative activity of the two preparations, as measured by the rate

TABLE XV

Specific Activity of Various Pepsins as Per Cent of Activity of Soluble Fraction

	Specific activity of soluble pepsin, average values	Specific activity of other samples as per cent of A′				
		Soluble fraction			Insoluble fraction	
Sample number		A′	39xx	44-61	44-9 crystals	46-33
Method						
Hemoglobin	0.33	100	100	98	74	64
Casein V⁻	750	100	100	95	100	100
					95	97
					110	125
					100	100
					(120)	110
			120		(120)	110
Casein non-protein nitrogen	0.37	100	100	121	107	
Gelatin V	6.0	100	100	110	90	
					135	140
					100	140
Rennet	330	100			120	85
					120	90

of digestion of various proteins, would differ; if the insoluble fraction were a mixture of the more soluble active protein with an insoluble inactive protein, the relative activity of the two preparations would be the same no matter what substrate was used. The relative activity of these preparations, as measured by several methods, is shown in Table XV. The figures show that the less soluble has about 70 per cent of the activity of the more soluble fraction, as measured by the digestion of hemoglobin, but the same activity, as measured by the digestion of casein, the rate of clotting of milk, or the change in viscosity of casein solution. The activity, as measured by the change in viscosity of gelatin solutions, appears to be slightly higher for the insoluble fraction.

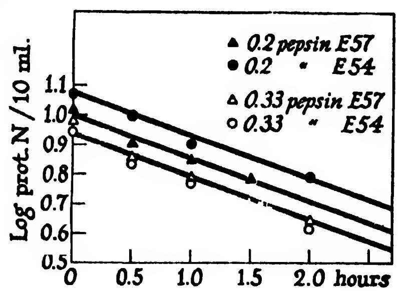

FIG. 11. Rate of inactivation of soluble and insoluble fractions at 66°C. pH 3.14, 0.1 M citrate buffer.

The variation in the relative specific activity of the two fractions indicates the presence of two or more active proteins in the insoluble fraction rather than the presence of an inert protein mixed with the soluble protein.

Rate of Inactivation.—The rate of inactivation of dilute solutions of the two preparations in 0.1 M citrate buffer pH 3.14 and 60°C. is shown in Fig. 11. No significant difference can be observed.

III. Solubility Curve of a Mixture of the Soluble and Insoluble Fraction

The solubility of mixtures of pure soluble pepsin with the insoluble fraction is shown in Figs. 12 and 13. Fig. 12 shows the effect of adding 3 or 10 per cent of the insoluble fraction to the pure soluble protein. It is evident that even 3 per cent of the insoluble fraction caused an unmistakable rounding of the curve even when the less precise method of amorphous precipitation is used.

The addition of 10 per cent of the insoluble fraction yields a curve closely resembling that of the original crude preparation. The curves are smooth and therefore indicate solid solutions rather than mixtures.

This fact is brought out more clearly in Fig. 13. In this experiment a series of solutions was prepared containing varying concentrations of the insoluble and soluble fractions but all having a total concentration of 1.7 mg. nitrogen per ml. The total protein concentration is therefore the same in all tubes while the proportion of the insoluble fraction varies from 0–100 per cent. The curve of the two preparations alone is also shown and the calculated curve expected if the proteins formed mixtures. Obviously the results are characteristic of a solid solution and entirely different from those expected if the proteins appeared as distinct solid phases.

IV. Preparation of Highly Active Pepsin from Pepsinogen

In connection with some solubility experiments on pepsinogen (Herriott, 1938) it was found that a small amount of protein was obtained which differed in certain properties from the main bulk of the preparation. Thus, when pepsinogen was fractionated as described in Table XVI a few per cent of the protein was recovered which had a much lower solubility and 2½ times higher "potential specific hemoglobin activity" than the starting material. This fraction (No. 12 of Table XVI) behaved in other respects like ordinary pepsinogen, *i.e.* upon acidification it rapidly changed into pepsin and about 15 per cent of its nitrogen was split off during activation and part of this nitrogen is a pepsin inhibitor (Herriott, 1938). Although highly active on hemoglobin this fraction has the same specific activity as

the bulk of the materials as measured with other substrates such as gelatin and milk.

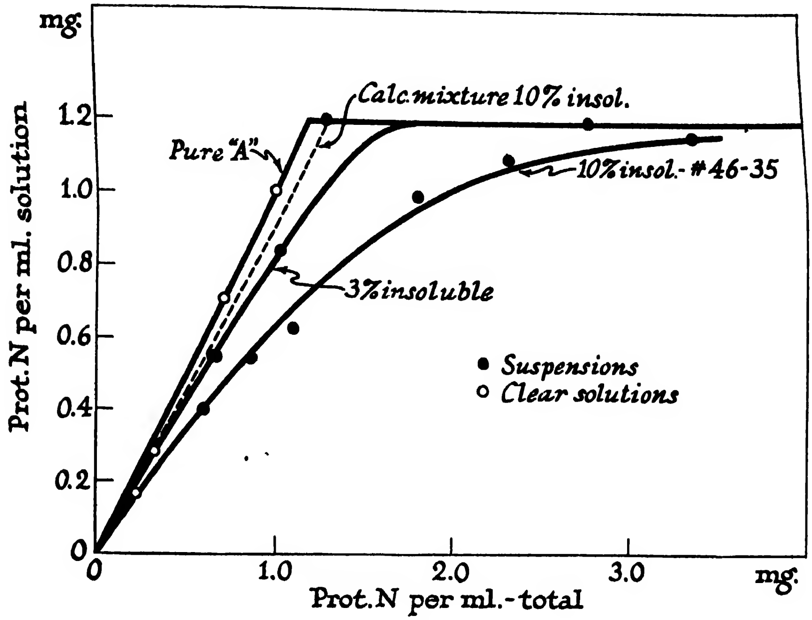

FIG. 12. Solubility of pure soluble pepsin mixed with increasing quantities of the insoluble precipitate 46-35 in the amorphous form in 0.5 saturated magnesium sulfate, 0.05 M pH 4.6 acetate.

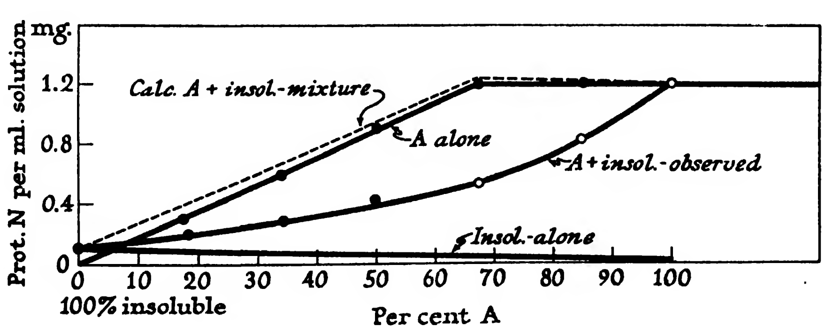

FIG. 13. Solubility of various mixtures of pure soluble pepsin and insoluble preparation in 0.5 saturated magnesium sulfate, 0.05 M pH 4.6 acetate. Total protein nitrogen concentration, 1.7 mg. per ml. in all cases.

A highly active preparation of pepsin could also be obtained by fractionation of pepsin prepared from ordinary pepsinogen. An example of such a

TABLE XVI

Preparation of "Highly Active" Pepsinogen

	No.	Volume	P.N./ml.	[P.U.]$^{Hb}_{mg.P.N.}$*
		ml.		
117 gm. 0.7 saturated ammonium sulfate filter cake of freshly prepared pepsinogen, dissolved and dialyzed against tap water at pH 6.5. 5 ml. M/1 di-potassium phosphate added to the protein solution to bring to pH 6.8, followed by 40 ml. M/1 pH 6.8 phosphate buffer; precipitate appeared and was filtered off and discarded. Filtrate diluted to 400 ml. and 400 ml. saturated ammonium sulfate added. Suspension....	1	900	2.4	0.22
No. 1 filtered. Filtrate.......	2	800	1.6	
Precipitate washed 5 times on funnel with solvent C (0.5 saturated ammonium sulfate, 0.05 M pH 6.8 phosphate). Precipitate....	3			
Precipitate dissolved in 15 ml. M/10 pH 6.8 phosphate and precipitated by 15 ml. saturated ammonium sulfate, filtered. Precipitate....	4			
Precipitate No. 4 treated as described for precipitate No. 3. Repeat. Precipitate...	5			
Precipitate No. 5 dissolved in 150 ml. pH 6.8 phosphate followed by 150 ml. saturated ammonium sulfate. Suspension....	6	300	1.2	0.24
No. 6 filtered and precipitate washed on funnel with solvent C and treated as for precipitate No. 3 through to precipitate No. 5. Filtrate and washings..........	7	350	0.8	
Precipitate plus 20 ml. pH 6.8 phosphate plus 20 ml. saturated ammonium sulfate. Suspension....	8	50	1.0	
No. 8 was filtered. Filtrate.......	9	50	0.17	0.45
Precipitate plus 2 ml. phosphate plus 2 ml. saturated ammonium sulfate, filtered. Filtrate.......	10	3	0.11	0.48
Precipitate plus 2 ml. phosphate plus 2 ml. saturated ammonium sulfate, filtered. Filtrate.......	11		0.08	0.49
Treatment repeated in No. 11, 3 times. Third filtrate..	12		0.06	0.54
Treatment repeated in No. 11, 3 times more. Third filtrate..	13		0.05	0.43
Precipitate from last treatment dissolved..........	14	3	0.84	0.12
Filtrates No. 9–No. 13 collected, precipitated with solid ammonium sulfate, filtered, and precipitate dissolved..........	15	8	1.5	0.44
No. 15 dialyzed..........	16			

* Pepsinogen has no proteolytic activity and the value in this column is the ratio of the proteolytic activity obtained after activation at pH 1.0–2.0, to the pepsinogen protein nitrogen or what has previously been called "potential specific activity."

fractionation may be seen in Table XVII. The procedure consists primarily of repeated dialysis at 5°C. and in a range of acidity from pH 3.5–4.3.

This highly active pepsin is much less stable than ordinary pepsin par-

ticularly near pH 2.0. Nearly 50 per cent of the highly active pepsin was destroyed in 15 hours at 25°C. as compared to only 5–10 per cent of the ordinary crystalline pepsin. It is estimated that the highly active pepsin is present as not more than 3-4 per cent of the original pepsin protein.

TABLE XVII

Preparation of "Highly Active" Pepsin from Pepsinogen

	No.	Volume	P.N./ml.	$[P.U.]^{Hb}_{mg. P.N.}$
		ml.		
Dialyzed pepsinogen	1	120	4.0	
No. 1 plus 30 ml. saturated magnesium sulfate plus 1 ml. 5 N hydrochloric acid, pH = 2.8. Solution dialyzed against 3 successive 1 liter aliquots of M/100 pH 3.5 acetate buffer for about 8 hrs. at 5°C. Centrifuged. Supernatant	2	110	1.8	0.3
Precipitate	2P			
Precipitate No. 2P dissolved in dilute sodium acetate, pH = 4.6	3	23	3.35	0.3
No. 3 diluted to 1.0 mg. P.N./ml. and titrated to pH 3.8; dialyzed at 5°C. against water, centrifuged. Precipitate	4P			
Supernatant	4	67	0.56	0.29
Precipitate from No. 4 dissolved in dilute acetate	5	23	1.3	0.37
No. 5 plus hydrochloric acid to pH 4.0; dialyzed 4 hrs. at 5°C., centrifuged cold. Supernatant	6	24	0.33	0.34
Precipitate	6P			
Precipitate No. 6P dissolved in dilute sodium acetate solution	7	20	0.74	0.37
No. 7 dialyzed at 5°C. for 15 hrs. at pH 4.3, centrifuged cold. Supernatant	8	25	0.15	0.33
Precipitate	8P			
Precipitate from No. 8P dissolved	9	10	0.72	0.40
At this point it was noticed a gelatinous precipitate appeared in solutions No. 6, 8, and 9, which resembled a certain type of rosette crystal plates. These suspensions were combined and centrifuged. Supernatant	10	43	0.26	0.31
Precipitate dissolved	11	10	0.25	0.46
No. 10 dialyzed at pH 3.8 and 5°C., a precipitate appeared, centrifuged, and precipitate dissolved	12	7	0.16	0.42
No. 11 dialyzed at pH 3.8 and 5°C., precipitate appeared, centrifuged, and precipitate dissolved	13	7	0.20	0.66
No. 13 after standing 2 days at 5°C.	14			0.49

Specificity of the Active Fraction.—If this active fraction represented a distinct enzyme, comparable to the gelatinase (Northrop, 1931), it would be expected to hydrolyze part of the protein molecule not attacked by the other fraction. This is not the case. Addition of the highly active frac-

tion to casein which has been previously hydrolyzed by the less active fraction causes no further increase in amino groups. Addition of the less active fraction to casein previously hydrolyzed by the active fraction likewise causes no increase in hydrolysis. Both fractions, therefore, attack casein at the same points.

It is interesting to note that the method of preparing the highly active pepsin from pepsinogen and the low active 0.1 $[\mathrm{P.U.}]^{\mathrm{Hb}}_{\mathrm{mg.\,P.N.}}$ pepsin from commercial products (Desreux, unpublished results) is practically identical; *i.e.*, dialysis at pH 4.0. No highly active pepsin was ever found in commercial preparations and, with the exception of solution No. 14 of Table XVI no low active fraction was found in pepsinogen pepsin.

DISCUSSION

The experiments reported show that pepsin prepared as originally described from commercial preparations or from pepsinogen contains two or more proteins. These proteins are indistinguishable by ultracentrifuge or electrophoresis but may be distinguished by solubility measurements. It is probable that more than one of these proteins is active but it does not necessarily follow that more than one pepsin occurs in any one animal.

It is quite possible that the insoluble fraction is a decomposition product of the more soluble fraction. In fact, heating a pure preparation of the soluble fraction results in a preparation which has a solubility curve very similar to the original crude pepsin.

The possibility that the protein originally came from different animals must also be considered. It is already known that pepsin from cattle is different from swine pepsin (Northrop, 1933*a*) and it is quite possible that the solubility method is sufficiently sensitive to distinguish between protein from different varieties of animals or between animals of different ages as well as between proteins from animals of different species (Landsteiner and Heidelberger, 1924). All the preparations used in this work represent extracts from a number of different individuals of different ages, varieties, and physiological condition. Attempts to prepare the enzyme from one individual were unsuccessful owing to the small amount present. It appears quite possible, therefore, that the enzyme may be homogeneous in any one animal but differs slightly in different varieties or age groups.

Analytical Methods

Nitrogen.—Total nitrogen was determined by the micro-Kjeldahl method.

Non-Protein Nitrogen.—1 or 2 ml. of the solution was added to 10 ml. boiling 2 per cent trichloracetic acid. The suspension was allowed to cool, filtered, and an aliquot of the filtrate analyzed for total nitrogen.

Protein Nitrogen.—Unless otherwise stated protein nitrogen was determined by meas-

urement of the turbidity of a dilute suspension in 2 per cent trichloracetic acid with a Klett photoelectric colorimeter of the Duboscq type. 1 ml. of the protein solution containing between 0.1 and 0.3 mg. protein nitrogen per ml. is added to 9 ml. boiling 2 per cent trichloracetic acid containing 25 ml. saturated magnesium sulfate per liter. The suspension is cooled to 20°C. and allowed to stand 1 hour. It is then compared with a standard suspension of known protein nitrogen content or with an M/25 copper sulfate solution in N/10 sulfuric acid which had been standardized against a known concentration of protein. The readings vary with the different pepsin fractions and for accurate results a separate standard solution must be prepared from the particular preparation analyzed. When standardized in this way the results are accurate to ±3 per cent.

Preparation of Solvents

Saturated Magnesium Sulfate.—A solution of magnesium sulfate in water having a specific gravity at 22°C. of 1.296.

Pepsin Activity.—Hemoglobin (Anson, 1938). The method was standardized against a glycerine solution of pepsin (Herriott, Desreux, and Northrop, 1940). On this scale 0.29 hemoglobin units per mg. protein nitrogen corresponds to Tiselius' value of 0.34.

Rennet.—(Herriott, 1938).

The other activity determinations were carried out as described by Northrop (1932).

SUMMARY

1. Solubility curves of crude pepsin preparations indicate the presence of more than one protein.

2. One of these proteins has been isolated and crystallized and found to have constant activity and constant solubility in several solvents.

3. The solubility measurements are complicated by the unstable nature of the protein and the fact that in certain solvents the solubility of the protein is markedly affected by the presence of non-protein nitrogen decomposition products while in others this is not the case.

4. A more insoluble protein has been prepared of lower solubility and lower activity, as measured by hemoglobin digestion. The activity, as measured by the digestion of other proteins, is about the same as the more soluble fraction. This insoluble fraction does not have constant solubility.

5. Mixtures of the insoluble and the soluble fractions give preparations having rounded solubility curves typical of solid solutions and resembling very closely those of the original preparation.

6. A small amount of pepsinogen and pepsin from pepsinogen has been separated which has nearly twice the enzymatic activity on hemoglobin as does the most active pepsin previously isolated.

REFERENCES

Anson, M. L., 1938, *J. Gen. Physiol.*, **22**, 79.

Butler, J. A. V., 1940, *J. Gen. Physiol.*, **24**, 189.

Desreux, V., and Herriott, R. M., 1939, *Nature*, **144**, 287.

Dyckerhoff, H., and Tewes, E., 1933, *Z. physiol. Chem.*, **215**, 93.
Herriott, R. M., 1938, *J. Gen. Physiol.*, **21**, 518.
Herriott, R. M., Desreux, V., and Northrop, J. H., 1940, *J. Gen. Physiol.*, **23**, 439.
Holter, H., 1931, *Z. physiol. Chem.*, **196**, 1.
Kunitz, M., 1938, *J. Gen. Physiol.*, **21**, 601.
Kunitz, M., and Northrop, J. H., 1938, *Compt.-rend. trav. Lab. Carlsberg*, **22**, 288.
Landsteiner, K., and Heidelberger, M., 1924, *J. Gen. Physiol.*, **6**, 131.
Northrop, J. H., 1930, *J. Gen. Physiol.*, **13**, 739.
Northrop, J. H., 1931, *J. Gen. Physiol.*, **15**, 29.
Northrop, J. H., 1932, *J. Gen. Physiol.*, **16**, 41.
Northrop, J. H., 1933, *J. Gen. Physiol.*, **17**, 173.
Northrop, J. H., 1933*a*, *J. Gen. Physiol.*, **16**, 615.
Northrop, J. H., and Kunitz, M., 1930, *J. Gen. Physiol.*, **13**, 781.
Philpot, J. St. L., 1935, *Biochem. J.*, *London*, **29**, 2458.
Steinhardt, J., 1938, *Proc. Am. Soc. Biol. Chem.*, **123**, cxv.
Steinhardt, J., 1939, *J. Biol. Chem.*, **129**, 135.
Tiselius, A., Henschen, G. E., and Svensson, H., 1938, *Biochem. J.*, London, **32**, 1814.

THE ELECTRICAL CAPACITY OF VALONIA

Direct Current Measurements*

By L. R. BLINKS and R. K. SKOW

(*From the School of Biological Sciences, Stanford University*)

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In a previous paper,[1] alternating current measurements were used to explore the nature of the large electrical capacity often exhibited by living *Valonia* cells. It was concluded that this possessed an appreciable "polarization" component, because it was not possible to balance the capacity of the cell at all frequencies against any single static capacity value in a simple series parallel network assumed to represent the cell circuit, over the audio frequency range employed. Instead, both the balancing capacity and its series resistance fell off with increasing frequency of alternating current. This is analogous to the capacity and resistance of a polarizable electrode (*e.g.* bright platinum in salt solution) in alternating current. However, not so pronounced a change with frequency occurred with cells as with electrode, and the actual capacity per square centimeter of cell surface was less. Accordingly it was suggested that the capacity was partly static, with an appreciable polarization component. This is consistent with the idea of a plasma membrane thin enough to act as a condenser, of fairly high resistance (*e.g.*, 10,000 ohms per sq. cm. of cell surface),[2] yet allowing sufficient passage of ions, with different relative mobilities, to set up diffusion potentials at rest, and presumably therefore to develop polarization under current flow.

Since the impaled cells are usually 1 to 3 sq. cm. in surface, and interpose but a single layer of protoplasm in series with the exterior and interior contacts, the actual capacity is large enough (1 or 2, up to even 8 or 10 microfarads), to permit transient measurements with direct current. The charge and discharge curves at make and break often occupy ½ second or longer, hence are readily recorded with a string galvanometer. Examples of these have been given in previous papers;[3] in the present paper they are

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[1] Blinks, L. R., *J. Gen. Physiol.*, 1935–36, **19**, 673.

[2] Blinks, L. R., *J. Gen. Physiol.*, 1929–30, **13**, 361, 793; 1930–31, **14**, 139.

[3] Blinks, L. R., *J. Gen. Physiol.*, 1935–36, **19**, 633

shown in balance against known capacities, with a view to indicating how closely they approach a static or polarization type. Metabolic and experimental control of the capacity is also described.

Methods

The Bermuda *Valonia macrophysa* was mostly employed, a few experiments with *V. ventricosa* from Florida yielding essentially similar results. Earlier work was done in Bermuda on freshly collected cells, but the more careful recordings were necessarily performed on cells brought from Bermuda to this laboratory. A variety of electrical and other criteria indicated that the cells were normal and healthy, and it is believed that the results are typical. Impaled cells were employed because electrical effects are best known in these, and current can be made to pass in only one direction across the entire protoplasm. The difficulties of distributed capacity[1] along the surface between two external contacts are also avoided. The cells were impaled in the customary manner, a fine glass tube (containing artificial sap) being inserted from above into the cell, which was supported on a glass ring. Several days were allowed for recovery after impalement, for the P.D. to stabilize, and for high resistance to appear. The latter is extremely variable, and depends among other factors, we now realize, on light. Thus a day or two of dim light or darkness was found to induce higher resistance, and prompt transient curves, whereas brighter light tends to abolish these, producing the state called "delayed polarization."[3] (The latter occurs only at rather high current densities passing inward across the protoplasm, and then only with a delay in its appearance, making the time curve sigmoid, and sometimes cusped.) This effect of light is best explained by the photosynthetic utilization of CO_2, the presence of which, as seen below, tends to raise the resistance of the cells, and makes for regular, prompt transients, with a more nearly static capacity. There is, however, an anomalous *initial* effect of bright light which at first *increases* the resistance and reactivity, before the later decrease mentioned. This agrees with other bioelectric,[4] as well as external electrode measurements[5] indicating initial anomalies in the acidity changes; these have been found in *Valonia* as well as in *Halicystis*.

The recording circuit was a simple D.C. bridge, similar to the one used in previous work,[2, 3] with an input arranged for reversal of current flow, and control of current density, from a battery of ten tapped dry cells and a potential divider. Ratio arms of 1000 ohms each were used for a 1:1 ratio, and a 10,000 ohm arm substituted for one of these to give a 10:1 ratio, often necessary when large cell capacities were encountered; (the ratio is in the opposite direction for capacities and resistances). General Radio resistance decades were used, sometimes supplemented by non-reactive resistances of higher value. A Leeds and Northrup type 1070 decade mica condenser, and two 1 μf. mica condensers of good quality were used to balance capacity. A Wagner earth ground, with capacity shunt, was used to balance out residual reactance in the bridge circuit itself; it was sometimes necessary to connect this across the detector, instead of across the input, presumably because of unbalances in the detector ground leaks (storage battery, etc.).

[4] Blinks, L. R., *J. Gen. Physiol.*, 1939–40, **23**, 495.

[5] Blinks, L. R., and Skow, R. K., *Proc. Nat. Acad. Sc.*, 1938, **24**, 413.

The detector and recorder was a single-stage vacuum tube amplifier, with a string galvanometer (Einthoven, or Kipp torsion string type) balanced into the plate circuit. Recording was on 35 mm. positive film, running at about 1 inch per second. The galvanometer response appeared essentially instantaneous to rectangular calibration curves at this speed (*e.g.*, see Fig. 3); and balances of one static capacity against another showed no deflection at make or break when the Wagner arm was properly adjusted (Fig. 2).

The calomel electrodes, through which connection to the cell was made by salt bridges, were of sufficient surface that no detectable polarization occurred with these alone (*e.g.*, using a dead cell as a control) at the current densities employed. In general the current densities here reported were low, usually not over 10 microamperes per sq. cm. of cell surface. Positive current passing outward across the protoplasm was usually employed, since resistance to this is a better indication of the "regular" state than to inward current, which often exercises a further "conditioning" effect raising the resistance while it flows.[3]

Types of Capacity Curves

It was originally intended[1] to record the charge and discharge curves of current flow across the protoplasm, with only a resistance balance in the adjacent bridge arm. Then this curve would be compared with a strictly exponential decay curve, calculated to fit it as closely as possible, or directly superposed over curves similarly recorded, taken with comparable networks of resistance and capacity, both static and polarization. The fit or the deviation would indicate the degree of approach to a static or polarization type of capacity respectively. However. variations in the speed of film travel, non-linearity of the galvanometer deflection toward the edges of its field, shrinkage of the film or print after development, etc., all made this method uncertain and tedious. Instead, since a network comparable to the cell had to be used in any case for comparison, this network was directly balanced against the cell in the bridge, and an immediate record of the deviations obtained over a range of capacity settings.

The principle of this is indicated in Fig. 1, where a family of exponential charge (or discharge) curves is shown, representing several different static capacity settings (C, $C/2$, $C/4$, $C/10$). When C is balanced against C in similar networks, of course no deflection results, but when C is balanced against $C/2$, etc., then the deflections of Fig. 1 *b* result. These are curves that start from the zero line, bend away, and approach it again, but never cross it. The closer one approaches to a balance, the less will be this deviation, until at balance a straight line results ($C - C$). If overbalanced, *e.g.* C *vs.* $2\ C$, then the curve deflects in the opposite direction, but it never crosses the zero line during its time course. Thus a balance can be approached from either side, with less and less deviation, until when balance

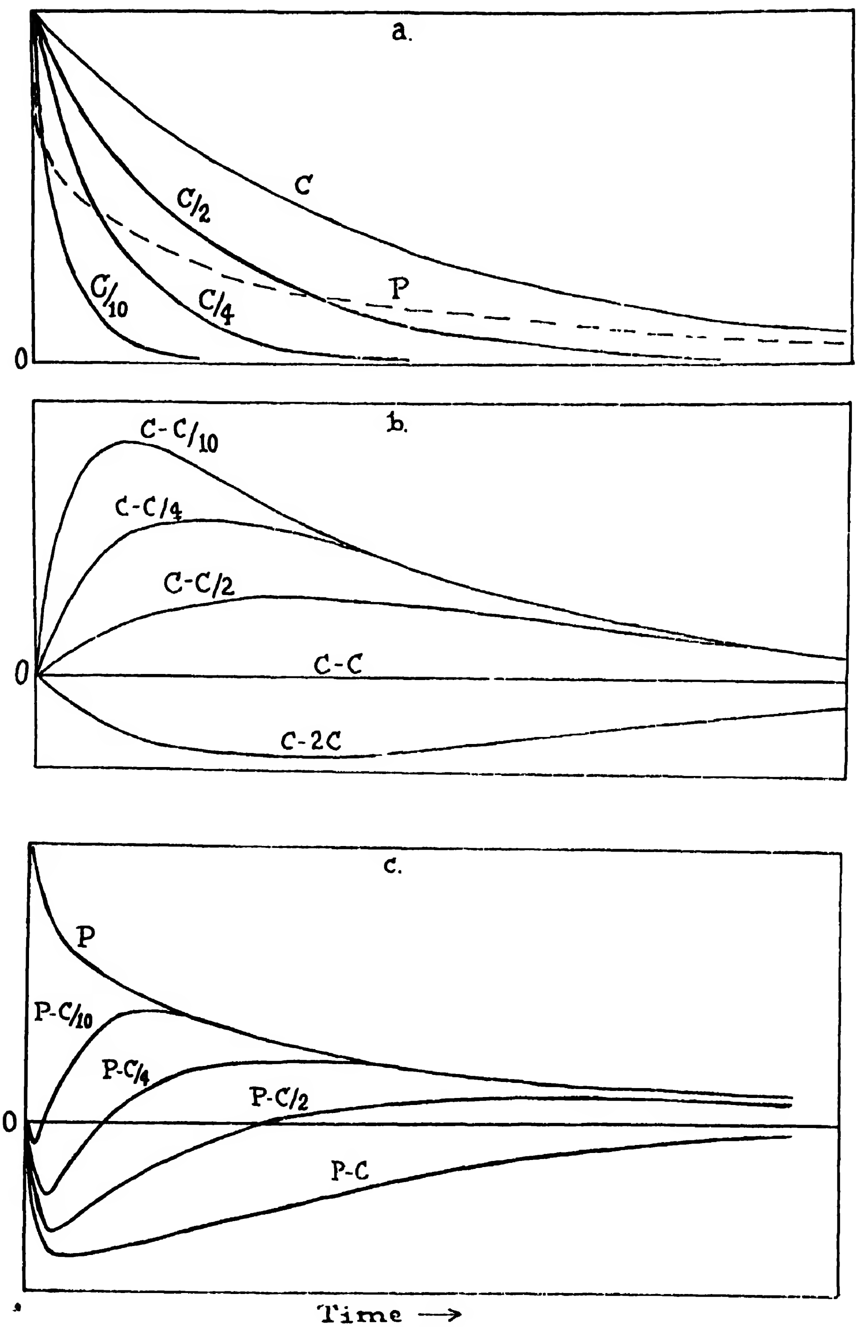

FIG. 1

is reached no deflection whatever results at make and break. An actual record of this sort is shown in Fig. 2, taken with two static condensers.

On the other hand, if we postulate a capacity (or resistance) increasing with time (which appears to be the case with polarization capacities), then the charge and discharge curves are no longer strictly exponential, but fall toward completion more and more slowly. Such a curve is indicated in Fig. 1 *a* by the broken line (P), which in turn cuts across the various exponential curves representing the static capacity charge or discharge. If this curve is subtracted from the several "C" curves, then the family of residual deflections of Fig. 1 *c* results. It is clear that no complete balance can ever be obtained. Instead, the lower C values produce first a deflection in one direction, then a return to zero (at the point where P cuts the given C curve), and a final deflection in the opposite direction, since P acts as if it were first smaller, then greater than the given capacity. With very large values of C, only the deflection below the line is found, now prolonged greatly. Clearly, no complete balance is possible over the entire time course with any single static capacity setting. A series of curves taken when a polarization capacity is balanced against a comparable network including a static capacity actually shows first a notched curve at lower C values, a definitely "diphasic" balance at higher C values, and finally a "monophasic" deflection with large capacities, but never a complete balance. An example is shown in Fig. 3, taken with a polarizable platinum electrode, balanced against a static capacity and resistance network. This should be compared with Fig. 2, where two static condensers balanced against each

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FIG. 1. Diagrammatic sketches of the time curves of charge (or discharge) obtained with static capacities, and an assumed polarization capacity. In (*a*) the exponential charge curves of static capacities C, $C/2$, $C/4$, and $C/10$ are shown, as well as an assumed polarization curve P, which has an effective capacity (or resistance) that increases with time, and therefore cuts across the whole series of exponential curves in turn.

(*b*) shows the effect of subtracting the charge curves of static capacities, which is accomplished when the transients are balanced against each other in a bridge. Thus C balanced by $C/10$ gives a cusped curve, due to the cutting off of the first, high part of the deflection, but thereafter following fairly closely the curve of C. $C-C/4$ and $C-C/2$ give successively lower curves, $C-C$ of course produces no deflection in either direction, while $C-2C$ produces one below the line.

(*c*) shows the effect of balancing P against the several exponential curves ($P-C/10$, $P-C/4$, etc.). The feature of the lower 3 C values is the cusp below the line, the balance curve crossing zero at the point where P cuts the given C curve in (*a*). Only with the largest C value, which P does not cut, is the residual curve entirely below the line, though with a different shape from that in (*b*).

It is understood that discharge curves are the same as these, merely inverted above and below the zero line.

other in comparable networks give only simple monophasic unbalance curves with too high or too low settings, and at balance yield a perfect straight line, with no notch or deflection in either direction.

With a mixed capacity, *i.e.* a static condenser in series or parallel with a polarizing electrode, intermediate types of curves result, with smaller residual diphasic elements appearing over the middle range of balancing

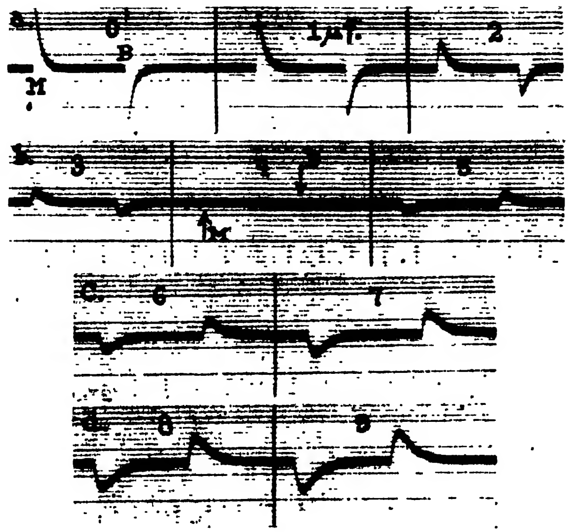

FIG. 2. String galvanometer records of transients due to charge and discharge of a 4 μf. static condenser balanced against static capacities of the values shown (in μf.). Note that there are no diphasic curves, the residuals becoming merely smaller as a balance is approached, and absolutely disappearing at balance (4 μf.). (Arrows show the moment of make, *M*, and break, *B*, with 4 μf. since there is no other indication.) With overbalance, monophasic residuals in the opposite direction result.

The capacities were included in a network involving a series resistance *r* of 48,144 ohms, and a parallel resistance *R* of 10,000 ohms (see Fig. 4) which are comparable to the values frequently met with in cells. Time intervals, shown by the faint vertical lines at the bases of the records, are 1/5 second.

values against a static condenser. Again no complete balance is ever possible, though the extent of the deviations is of course less than with a pure polarization capacity. This mixed capacity more closely resembles the cell behavior, toward which we may now turn.

Cell Recordings

In recording and balancing the charge and discharge curves of living cells, proper resistance as well as capacity settings must be made. The network assumed for this purpose is that of Fig. 4, with the corresponding

cell components diagrammed beside it. The network is admittedly oversimplified, since it lumps the entire protoplasmic complex, with its almost certain two capacities in series (at the outer and vacuolar cell surfaces),

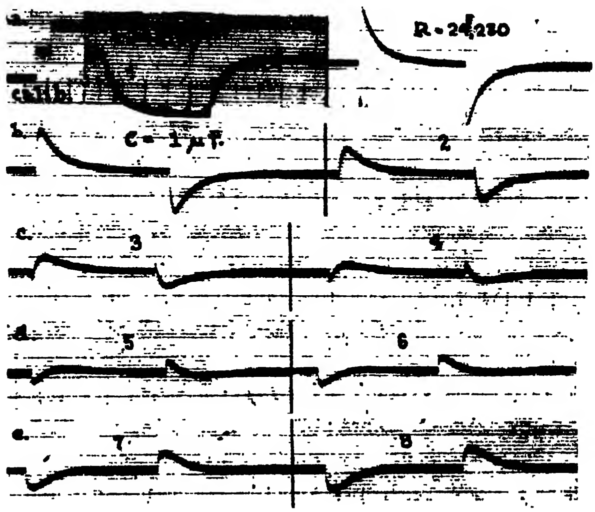

FIG. 3. String galvanometer deflections resulting from the make and break of current through a pair of small platinum electrodes in 0.5 M KCl solution, wired into a circuit containing a series resistance r of 46,190 ohms, and a parallel resistance R of 24,230 ohms, the electrodes representing C, as in the network of Fig. 4.

In (a) is first shown a calibrating potential of 20 mv. (+ and −) indicating the essentially rectangular deflection of the galvanometer at this speed of recording. Next in (a) the polarizing electrode is balanced against only the series resistance r, giving a deflection curving away from zero at make, and returning to it at break. Finally in (a), R is also introduced, so that the deflection is first away from zero, and then returns to it in the steady state, both at make and break.

In the remaining records, C is introduced, with equivalent values of 1, 2, etc. μf. up to 8 μf. in the final record. It is clear that no setting gives a complete balance. With 2 μf. sharp cusps are evident carrying the curve abruptly to one side of zero before the opposite deflection occurs; these diphasic curves are most clear with 3 and 4 μf., while with 5, 6, and more μf. the residual curve becomes almost entirely monophasic in the direction of these cusps.

Actual bridge ratio, 10:1, the effective resistance and capacity values being corrected for this. Time intervals, 1/5 second, shown by the faint vertical lines at the bases of the records.

and probably other resistance and capacity elements at the plastids, nuclei, and other cell structures. However, since it is impossible to assign individual values to these, it seems best to assume the simplest possible three element network. Some justification may be found for this, since, as seen below, it approximates to almost perfect balance under some conditions.

r is the sum of all those resistances in series between the electrodes, up to, but not including, the protoplasm itself; in practice it is largely due to the narrow sap-filled impaling capillary inserted into the vacuole. It usually lies between 10,000 and 50,000 ohms, higher resistances being inconvenient for this and other purposes (*e.g.*, recording with the "Micromax" potentiometer). A good value is 25,000 to 30,000 ohms. This can be determined in advance of impalement with the sap-filled capillary, and the value does not vary greatly from this, except with temperature, or concentration changes within the tube above the capillary (due to evaporation, or diffusion of KCl from the upper salt bridge). It also accounts for most of the resistance of the living cell, when the latter is in its truly "delayed" polarization state, at current densities too low to induce high protoplasmic resistance. However, when cells are in the "regular" state, which is necessary for these measurements, other means have to be taken to check the value of r. One method is to measure with alternating current at the higher audio frequencies (*e.g.* 10,000 cycles); here the impedance of the protoplasm is very low, and the total impedance is practically equal to r.[1] But it can also be done by inspection, or by recording, of the string galvanometer deflections at make and break. Fig. 5*c* indicates the criteria of such balance: at make, a smooth immediate start of the charging curve from zero (or other reference line if a P.D. is displayed), without either a momentary cusp in the opposite direction (Fig. 5*d*, *e*) or a jump away, leaving a blank space before the charging curve begins (Fig. 5 *a*, *b*). The latter (*a*, *b*) indicates that r has been set too low; the former (*d*, *e*) that it is too high. The flickers produced by the cusps (*d*, *e*) are easier to detect by the eye than the jumps (*a*, *b*), and in practice the balance is approached from this, the high side, until they just disappear. Actual recording was performed in critical cases, and the best value chosen after development of the film.

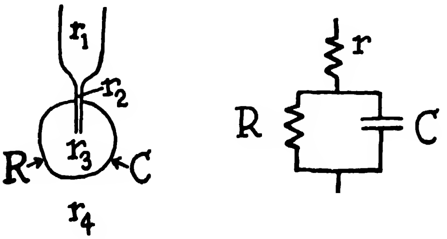

FIG. 4. Diagram of the impaled cell of *Valonia*, indicating its component resistance and capacity elements. r_1, r_2, etc. represent the series resistances leading up to the protoplasmic surface; these are largely located in the narrow impaling capillary (r_2). R and C are the parallel resistance and capacity respectively of the protoplasm as a whole. In the right hand diagram is the electrical network assumed to represent the cell, and to balance it in the bridge. Under some conditions, it gives a nearly perfect balance.

With r thus established, R, the effective D.C. resistance of the protoplasm is then readily determined by the setting which brings the curve back to zero (or to the P.D. reference line) after the charging transient is over, as in Fig. 5*f*. This adjustment simply shifts the charging curve downward by the amount representing the former off-balance due to R; its shape is not changed, and it merely starts *below* the line to the extent of its former steady state deflection *above* the line. (The discharge curve remains in its same position throughout all the adjustments of Fig. 5.) The value of R necessary to make this steady state balance is from 5,000 to 20,000 ohms in most cells displaying regular transients. It tends to become higher under the conditions favoring static capacity, although this is not always the case.

R being set, we may now attempt to balance *C*, in parallel with *R* in the network (and presumably in the cell). Fig. 6 shows a series of records of

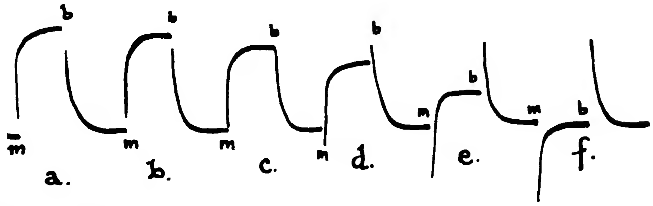

FIG. 5. Sketch indicating the criteria of resistance balance, employing the network of Fig. 4, but with a capacity setting of zero. *a* and *b* represent too low values of *r*, since the transient curves do not start smoothly from the base line, but exhibit a clear space or jump at both make and break. *c* represents a proper balance of *r*, since no such break in the curves appears. *d* and *e* represent too high values of *r*, shifting the charging curve downward on the record; as a result, small cusps below the base line appear at make, and above the steady state deflection at break. Finally, if *r* is correctly set according to this test, and *R* is then adjusted so that the steady state of the charging curve is brought to the base line, curve *f* results. The capacity *C* can then be adjusted in turn.

It should be noted that only the position of the charging curve is shifted by these adjustments, the discharge curve remaining in the same position throughout.

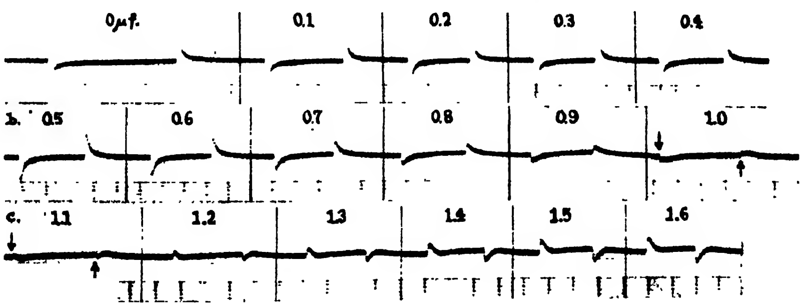

FIG. 6. Balance of D.C. transients at make and break of current through a *Valonia* cell. *r* and *R* being balanced, *C* was varied from 0.0 μf. to 1.6 μf. as shown (the cell values were 10 times this high due to a 10:1 ratio in the bridge). It is clear that a balance is being approached at close to the 1.0 or 1.1 μf. setting, but that even here there is a residual diphasic deflection, which is also evident with 1.2 and 1.3 μf. settings, which deviate more and more from balance. No single setting gives complete balance of the transients.

Bridge ratio, 10:1, making the actual cell capacities 10 times those marked. Cell surface about 3 sq. cm., giving at the best setting, 3 to 4 μf. per sq. cm. of cell surface. r = 60,000 ohms, R = 20,000 ohms. Time marks at base of record, 1/5 second apart. Current density about 10 microamperes per sq. cm. surface (outward flow).

this sort, taken with a cell which reacted promptly and regularly, as seen in the initial, purely resistive balance. With increased values of *C*, the

deflections at charge and discharge become notably reduced, but display a diphasic type of curve at medium C values, passing over into deflections to the opposite side of zero, at higher C values. Nowhere is there a complete balance attained although it is considerably better than that given by a polarizing electrode similarly balanced against a condenser (Fig. 3). This

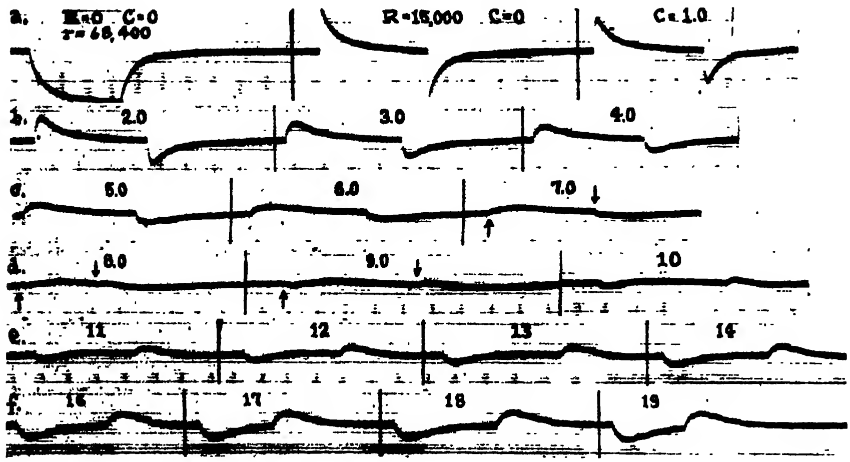

FIG. 7. Transients due to make and break of current through impaled *Valonia* cell. The first curve is that with r only in the balancing arm, giving a smooth deviation from zero at make, and a return at break. R is next introduced, shifting the charging curve upward so that it returns to zero in the steady state, the discharge curve remaining unchanged. C is then introduced in parallel with R, with the effective values in microfarads as marked. The charge and discharge curves become progressively smaller with increasing C values, until at 8 and 9 microfarads there is close approximation to a balance. There is however, a residual diphasic unbalance even here, giving a deflection first in one direction, then in the other, indicating a degree of polarization element unbalanced by the static balancing capacity. (Make and break here indicated by arrows.)

Actual bridge ratio, 10:1, but with values here corrected to read for the cell as marked. $r = 68{,}400$ ohms, $R = 15{,}000$ ohms, C at best balance, 8 to 9 μf. Surface of cell about 3 sq. cm. Current density, about 10 microamperes per sq. cm. of cell surface. Time intervals, 1/5 second, shown by the faint vertical marks at the base of the records.

is consistent with the cell's displaying an appreciable degree of polarization, which cannot be balanced completely by a static condenser.[6]

[6] It should be understood that other causes beside polarization might produce deviations from a strictly static capacity. Thus a poor paper condenser often does not balance against a good mica one over the entire charge and discharge time curve, or over the frequency range in A.C. (See for example Laws, F. A., Electrical measurements, New York, McGraw-Hill, 1917, 363–364; Grover, F. W., *Bureau Standards Bull.*, 1911, **7**, 495.) Such imperfect dielectrics might well occur in the cell surface. But it is possible that ionic "impurities" may be responsible for the deviations in both cases.

On the whole, this is the type of record generally given by *Valonia* cells. Another example is shown in Fig. 7. This is quite aside from even more complicated curves sometimes obtained, in which, for example, the charging curve is balanced at one capacity, but a different setting must be found for discharge. This is probably due to the "conditioning" or "deconditioning" effect of current flow as such, which has been described in a previous paper. Fig. 8 shows an example of this unsymmetrical state.

However, from time to time cells come into a state of more or less complete balance by a proper value of static capacity, where only a slight flutter at the very instant of make or break may betray a momentary unbalance—so rapid as to be off the reliable range of the D.C. transient recording. In general, the capacity required for such balance is also appreciably smaller than that giving the nearest approach to balance in the polarization type.

In an endeavor to control this occurrence of static capacity, changes in the acidity of sea water were made, since earlier work had shown that more regular transients, and higher resistances, were induced by acidification of the sea water, especially by weak acids, such as CO_2 and acetic acid.[3] Added to the sea water until the pH by an indicator was about 6.0, these acids were indeed found to be most effective in shifting the capacity toward the static type. (Strong acids such as HCl are also effective but require a longer time.) Fig. 9 indicates the high degree of balance attained against a static capacity when such acidified sea water was given to a cell previously displaying a good degree of polarization capacity (*e.g.*, as in Fig. 7). On returning the cell to ordinary sea water, this induced static capacity persists for some time—several hours or even overnight—then slowly regains more of a polarization component. Loss of the static character, or even of any polarization at all, on the other hand, is favored by the addition of traces of penetrating base, such as ammonia, to the sea water. As shown earlier,[3] this tends to lower the resistance, and abolish all polarization, except to very large inward currents, where a typical delayed polarization finally appears—presumably because the inward current overcomes the ammonia effect, perhaps by increasing the internal acidity.

Other influences which tend to produce partial or complete attainment of a static type of capacity include low oxygen tension, low temperatures for a considerable length of time, and a variety of chemical substances such as phenols, cresols, quinones, cyanide, and other narcotics, etc. It is suggestive that most of these agents also produce a positive P.D. in *Valonia*. Some of them may have a specific action on the cell surface; nevertheless, the similarity of their behavior suggests that they may all be operating in the same manner, namely by increasing the internal acidity of the protoplasm, possibly by favoring fermentation and other anaerobic processes increasing

acid production in the plant cell. This hypothesis needs further testing, but is favored by the fact that ammonia can often counteract many of

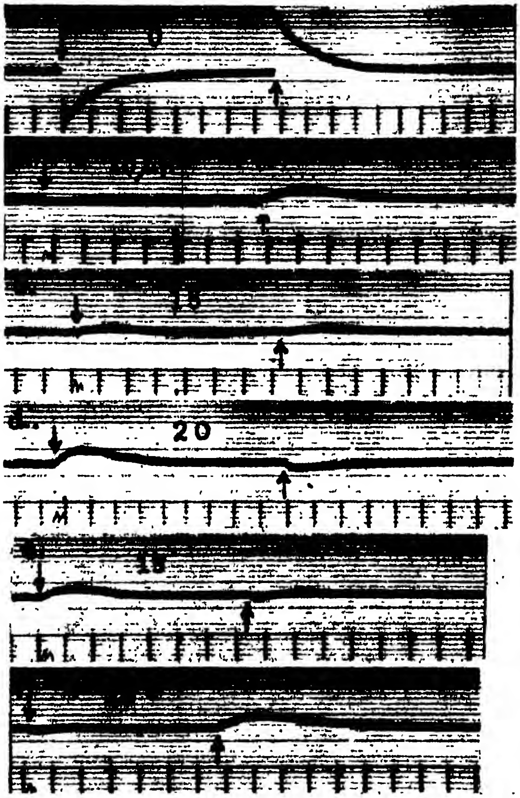

FIG. 8. Records of current flow in impaled *Valonia* showing an asymmetry between the charge and discharge curves. In *a*, the charge and discharge curves alone are shown, compensated as to r and R, but not as to C. Then the balance with 10 μf. is shown, which is almost perfect as to make, but still unbalanced in the break curve. Increase to 15 μf. produces two nearly equal, slightly diphasic curves, but both curved upward. 20 μf. badly overbalances the charge curve, but only slightly overcompensates the discharge. Return through 15 μf. to 10 μf. repeats the earlier records. It is clear that a different capacity value governs the time course for charge and discharge, and that the charging curve is more nearly compensated by a static condenser than is the discharge.

R_a = 20,000 ohms, r = 60,000 ohms. Surface of cell about 2 sq. cm. Outward current about 10 microamperes per sq. cm. Time marks at base of records, 1/5 second apart.

their effects, although some of the agents seem to be immune to, or even more powerful, than ammonia.

Light, as mentioned earlier, also influences the type of capacity, which tends toward the static type when the cells have been kept dark for some time. This is presumably due to the internal accumulation of CO_2 and possibly other acids. Illumination produces more polarization character-

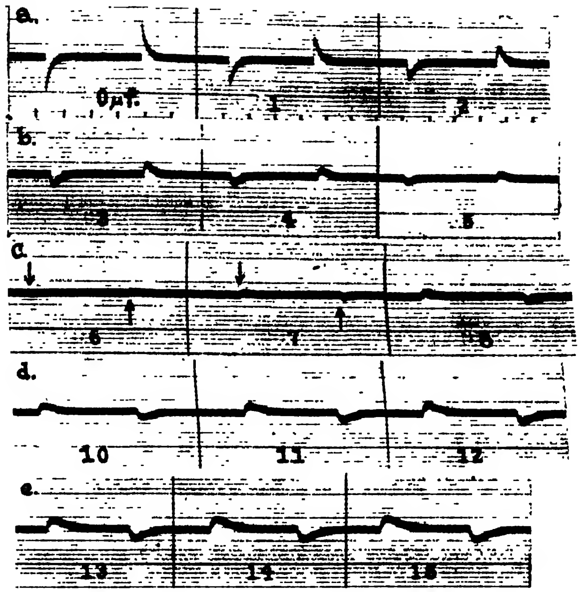

FIG. 9. Transient records of make and break of current through impaled *Valonia* cell which has been exposed for a short time to sea water, acidified with acetic acid to a pH of about 6.0. Smooth, symmetrical charge and discharge curves are seen against increasing balancing capacity, and when the latter reaches 6 μf., an almost perfect straight line results at make and break in record *c*. (Arrows indicate make and break moments.) Higher *C* values cause only symmetrical deflections, without diphasic component. The entire record closely resembles Fig. 2, the balance of two static capacities, and it is to be concluded that the cell's capacity is likewise entirely static in this case, and about 3 μf. per sq. cm. of cell surface.

Cell surface about 2 sq. cm.; outward current, 6.5 microamperes per sq. cm. of cell surface. r = 60,000 ohms, R = 5,000 ohms. C values in μf. as marked. Time intervals at base of record *a*, 1/5 second apart; same record speed throughout.

istics if long continued, but there is, during the first 5 or 10 minutes of illumination, often an enhanced resistance, and a distinctly more static capacity, than during the later course of illumination. This corresponds with other evidence, mentioned above,[4, 5] that there is an anomalous increase of acidity as the first effect of light, which precedes the later alkaline drift as CO_2 is consumed. There is a great deal of evidence, still unpublished, that this occurs in *Valonia*, just as it does in *Halicystis*, and accounts

for remarkable cusps of the P.D. under certain conditions. The effect upon resistance and capacity, in giving results like those of increased acidity, still further increases the evidence for the reality of this anomalous effect.

DISCUSSION

It is to be concluded that the electrical capacity displayed by *Valonia*, and possibly by other cells now under study (*e.g. Halicystis*, *Nitella*) is not rigidly of one sort, but naturally ranges from strictly static, to increasing deviations therefrom. It is under experimental and metabolic control; conditions which increase the internal acidity of the protoplasm, tend to raise the resistance, lower the capacity, and render the latter more strictly static. All of these changes are in agreement with effects upon the bioelectric potential itself, and with the response of the latter to changes in the ionic environment. For weak acids, as well as agents similarly affecting the P.D., also greatly alter the response to dilution of sea water, substitution of K for Na, and of NO_3 for Cl, etc.[4] Such effects on *Halicystis* are paralleled by some on *Valonia*, shortly to be published. We may therefore conclude that the cell surface can take on varying degrees of ionic permeability: (1) It may permit relatively free passage to all ions, in which case its resistance, P.D., and polarizability are low; (2) it may become somewhat less permeable to one species of ion than to another, in which case its resistance increases, its P.D. responds well to ionic concentration changes, and it develops a counter-E.M.F. of polarization type; and (3) it may become relatively impermeable to all ions, in which case its resistance is very high, its P.D. less responsive to some ionic alterations, and its capacity nearly or entirely static, the thin, poorly conducting cell surface becoming a condenser. The first condition is that of an electrolytic conductor, the second like a polarizing, partially reversible electrode, the third like a mica or oil condenser.[6]

This concept of a variable cell surface is perhaps disturbing to a fixed, definitive description of its properties, but is in accord with a great many recent findings, which relate its properties to metabolism and other experimental conditions. It seems necessary that any speculations as to the nature of the cell surface must include, among other things, its remarkable lability. Whether a strictly lipoid surface will display these properties, or whether a more complicated structure must be assumed, will depend upon studies of comparable models, such as liquid phases, and multi- or monomolecular layers.

SUMMARY

Impaled cells of *Valonia* were balanced in a Wheatstone bridge against a simple series-parallel circuit of two resistances and a capacity, the transient charge and discharge curves at make and break of direct current being recorded with a string galvanometer. With the resistances properly balanced, a series of characteristic deflections resulted when the balancing capacity was varied. With many cells, no complete capacity balance was ever attained over the entire transient time course; but instead either a monophasic or diphasic residual deflection always remained. This behavior is comparable to that of a polarizing electrode in D.C., although not so clearly marked; and it is concluded that *Valonia* usually has an appreciable polarization component, probably in parallel with a static capacity.

However, some cells can be balanced almost completely against a mica condenser of proper value, which indicates that they display a nearly pure static capacity under some conditions. This static state could be produced experimentally by exposure to weak acids (acetic, carbonic, etc.) and by metabolic agents probably inducing internal acidity (low oxygen tension, long exposure to cold, narcotics, etc.). Conversely, penetrating weak bases, such as ammonia, abolished the static capacity, or even any regular polarization. Light acts something like ammonia, after an initial "acid gush" anomaly. Most of these agents likewise affect the P.D. and its response to external ionic alterations, and it seems likely that the change in capacity type reflects altered ionic permeabilities and relative mobilities.

THE EFFECT OF UNILATERAL ULTRAVIOLET LIGHT ON THE DEVELOPMENT OF THE FUCUS EGG*

By D. M. WHITAKER

(*From the Department of Biology, Stanford University*)

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INTRODUCTION

Kniep (1907) and others observed many years ago that several species of *Fucus* eggs form rhizoids on the least illuminated sides when they are illuminated from one side by white light. The plane of the first cell division, and the developmental axis, are also determined. Winkler (1900) and Knapp (1931) have shown that *Cystosira* eggs respond in a similar manner. Hurd (1920) investigated the effects of different regions of the visible spectrum on the eggs of a species of *Fucus*, then known as *F. inflatus*,[1] from San Francisco Bay. She used Wratten filters to obtain violet, blue, green, yellow, and red light from sunlight and from arc sources. A large proportion of the energy transmitted by these filters was contained in a fairly narrow span of wave-lengths. The intensity was measured, and in some experiments it was equal throughout. Hurd found that the place of rhizoid origin was determined only by the shorter wave-lengths; *i.e.*, by the violet, blue, and probably by the shorter green (λ4000 to 5200 or perhaps somewhat more Ångstrom units). Some evidence that ultraviolet might be effective is referred to in the summary, but no results or information are given, and investigation of this region of the spectrum does not appear to have been pursued. The longer wave-lengths of the visible spectrum, 5800–7000 Å, had no effect even when the intensity was relatively high. The same wave-lengths that determined the place of rhizoid origin (4000–5200 Å) also caused negative geotropism of the growing rhizoid.

A number of other factors may also determine the place of rhizoid origin. These factors, and some of their interrelations, have recently been reviewed (Whitaker, 1940 *a*), and therefore at present they will merely be listed. They include the presence of neighboring eggs (Kniep, 1907; Hurd, 1920;

* This work has been supported in part by funds granted by The Rockefeller Foundation.

[1] Probably *F. nitens* in Gardner's (1922) more recent classification, and similar to *F. furcatus*.

Whitaker, 1931), especially in acidified medium (Whitaker, 1937 *a*; Whitaker and Lowrance, 1940); diffusion gradients resulting from development near one end in a capillary tube (Whitaker, 1937 *b*); electric current (Lund, 1923); pH gradients (Whitaker, 1938); temperature gradients (Lowrance, 1937); stratification by centrifuging (Whitaker, 1937 *c*, 1940); artificially imposed elongation (Whitaker, 1940 *b*); gradients of beta-indole acetic acid (Olson and Du Buy, 1937).

The experiments now to be reported were undertaken to test the effects of unilateral monochromatic ultraviolet light.

Material and Method

Fucus furcatus was collected at Moss Beach and at Pescadero Point, California, and gametes were obtained by methods which have been described previously (Whitaker, 1936). The eggs are somewhat variable in size, ranging from 65 to 90 μ (average 75 μ) in diameter. Experiments were carried out from January to May, inclusive, 1940. This species of *Fucus* is hermaphroditic and fertilization takes place when the capsules, each containing eight eggs, dissolve and liberate the eggs into sea water in which sperm are already swimming. The dissolution of the capsules can readily be observed under the microscope, and eggs were selected which had been fertilized during a span of 10 minutes. The midpoint of this span is the time of fertilization $\pm$ 5 minutes. Eggs were shed and fertilized in filtered sea water (specific gravity, 1.026–1.027), at pH 8.0–8.3, in the dark or in red light in a constant temperature room at 15 $\pm$ $\frac{1}{4}$°C.

Eggs were reared in the constant temperature room approximately until the time of irradiation. After irradiation they were kept in a constant temperature cooled incubator, which was usually at about 15°C. During the irradiation the temperature approximated 15°C., although the system of control was not precise in this case. In all cases the eggs were constantly shielded from light which affects the rhizoid formation, except for the experimental exposure to ultraviolet, until the final results were observed. Immediately after fertilization the eggs secrete a pecten or cellulose-like jelly which gradually hardens to form a firm but sticky investing cell wall. By about 2 hours or somewhat longer this material attaches the eggs quite firmly to the bottom of the dish so that they do not roll or move if carefully handled. It was, of course, essential to minimize movement of the eggs after the irradiation, since there are no visible markers or points of reference on the spherical eggs. The culture vessels were handled with great care and were kept in the incubator in a levelled moist chamber mounted on sponge rubber to reduce vibration. The results were usually recorded at about 24 hours after fertilization, when well developed rhizoid protuberances are present. In certain cases after strong irradiation, however, the development was considerably delayed.

The eggs were grown and irradiated in six culture vessels made of clear fused quartz 1 mm. thick. These vessels were made in the form of cubes, 1 cm. $\times$ 1 cm. $\times$ 1 cm., with open tops. Two opposite sides of each vessel were polished on the outside. The eggs were spaced thinly over the square centimeter of vessel bottom and were irradiated from the side so that the direction of rhizoid formation could be observed from above. No eggs were counted which were within 5 egg diameters of another egg, or of a vessel wall, and most eggs were considerably further apart. A lip pipette was used to space the eggs.

Although the quartz vessels were well made and the sides were quite well aligned, fused quartz is difficult to work and, as the results ultimately indicated, these dishes were not optically perfect nor did they all deliver identical doses to the eggs on the bottom. This was true even when the eggs were thinly and similarly spaced. There was inevitably a certain amount of eclipsing in the populations irradiated from the side, although the incident beam slanted downward approximately 3° to reduce the eclipsing. In a given set of experiments the eclipsing, like most other factors, was essentially similar throughout. It is hoped to obtain vessels of a different design for future work and to know precisely the dosage received by each egg. In the present experiments the absolute intensity of the beam reaching the face of the culture vessel is known quite precisely, but the dose actually reaching the eggs in the populations is known only relatively. It must also have varied somewhat for different individual eggs in a population, although differences in response in different regions of the bottom of the vessel were small. Some vessels consistently gave somewhat greater response than others. This was probably due largely to differences in dosage received by the eggs due to different optical properties in the region of junction of the bottom and side of the vessel. Such differences were not great compared with those resulting from the differences in applied dosage, and they tend to cancel out when the results of different experiments are averaged.

The total energy applied to the side of the culture vessel, and the loss in passing through 1 mm. of flat, polished quartz into sea water, are known quite precisely. The absorption of λ2804 Å by the sea water in the culture vessels was found by direct measurement to be negligible. If the sides of the culture vessels were optically perfect, and if there were no eclipsing, the unit of dosage used in this paper would represent 1.2 ergs per mm.^{2} applied to the eggs. In view of these two undetermined correction factors, however, 1.2 ergs per mm.^{2} must instead be regarded as the order of magnitude of the energy actually applied to the eggs.

Dr. Arthur Giese kindly permitted use of his ultraviolet equipment, which attains unusually high intensity and monochromatic precision. The source was a large mercury arc operated on 3–6 amperes of direct current supplied by a 220 volt generator. Special devices maintained a relatively constant current. After passing through a slit, the beam passed through a monochrometer with two large quartz crystal prisms, and lenses, which separated the bands of different frequency. To further purify, the selected band was admitted through another slit and passed through a second monochrometer with lenses and a single large fused quartz prism. At the site of irradiation the emerging monochromatic beam was elliptical in cross-section and large enough, with a convenient margin, to cover the entire side of a culture vessel with very nearly uniform intensity. The rays were nearly parallel but diverged slightly. The intensity of the ultraviolet light was measured by means of a sensitive thermopile and a high sensitivity galvanometer. The thermopile was made by Mr. Emerson Reed. It was blackened with zinc black, which absorbs all frequencies to the same high degree.

RESULTS

It has been shown earlier (Whitaker and Lowrance, 1936) that the response of a population of *Fucus* eggs to unilateral illumination by white light from a frosted 40 watt electric lamp at 1 meter depends on how long

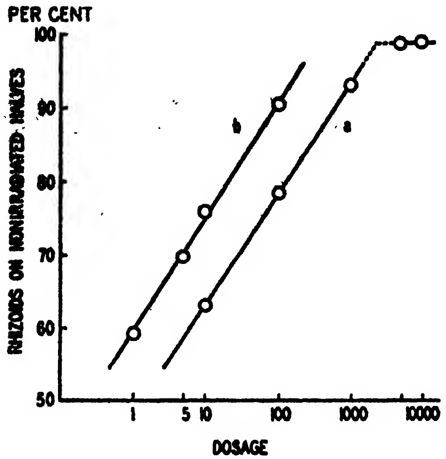

FIG. 1. Curves *a* and *b* show the results of two different series of experiments carried out at different times of the year and under somewhat different conditions (see text). The vertical axis indicates the percentage of eggs which formed rhizoids on the halves away from the source of radiation (λ2804 Å) after unilateral irradiation. The horizontal axis shows the dosage on a logarithmic scale. 1 unit of dosage represents the application of 1.2 ergs per mm.² to the side of the culture. This is not necessarily the amount of energy received by each egg (see text).

Curve *a* shows the results of experiments carried out in January. Each point represents the average of six to seven experiments involving a total of 354 to 461 eggs. Rhizoids were inhibited by a dosage of 20,000 units.

Curve *b* shows the results of experiments carried out in March. Each point represents the average of twenty experiments involving a total of 1529 to 2200 eggs.

the eggs have been fertilized. Thus exposure for 1 hour beginning 2 hours after fertilization (15°C.) had almost no effect, while a similar exposure beginning 7 hours after fertilization caused 97 per cent of the eggs in a population to form rhizoids on the side away from the light. The response was nearly maximal throughout the period 7–11 hours after fertilization. The rhizoid protuberances do not begin to form until some time later, 12–24 hours (average 18) after fertilization (Whitaker, 1936).

In view of the results with white light, the first exposures to ultraviolet were started between 7 and 10 hours after fertilization. After preliminary results had shown that high percentages of the eggs responded, the magnitude of the response was studied with respect to two variables: the amount of radiation, and the time after fertilization when it was applied.

The First Series of Experiments

The first series of experiments was carried out through most of January, 1940, using λ2804 Å. The dosage (total energy) applied to populations was 10, 100, 1000, 5000, 10,000, and 20,000 units. The intensity of the beam did not vary greatly in the different experiments and the dosage was varied principally by varying the duration of exposure. The time required to apply the various dosages was usually approximately as follows: 10 units, 7–7.5 seconds; 100 units, 70–90 seconds; 1000 units, 11½–15 minutes; 5000 units, 55–62 minutes; 10,000 units, 1 hour 55 minutes–2 hours 8 minutes; 20,000 units, 4–5 hours.

The results of this first series of experiments are shown graphically in Fig. 1, curve *a*. Each point is the average of either six or seven experiments involving a total of 354 to 461 eggs. When no radiation at all is applied, the rhizoids form in random directions so that 50 per cent form on the halves of the eggs away from either side of the vessel. The effect of the radiation is therefore indicated by the excess above 50 per cent which form rhizoids on the halves away from the source of radiation. The total energy applied is shown on the horizontal axis on a logarithmic scale. It is clear that the curve is approximately a straight line over most of the range of response, from low to near maximal. At both 5000 and 10,000 units the response was maximal; slightly more than 98 per cent of the eggs formed rhizoids away from the source of radiation. The percentage was 100 in half of the populations irradiated with 5000 and 10,000 units. After strong irradiation, especially after 10,000 units, the rhizoids of most of the eggs formed very nearly opposite the source of the ultraviolet light, as shown in Fig. 2.

In three experiments of the first series, cultures were irradiated with 20,000 units. The irradiation began at approximately 8 hours after fertilization and lasted 5 hours in two cases and 4 hours in the other. In the first two cases no rhizoids whatsoever developed, while in the third case 22 out of 165 eggs formed rhizoids, mostly delayed, and the remainder formed no rhizoids. None of the eggs which failed to form rhizoids were cytolized. It appears, however, that this dosage is almost completely inhibitory to rhizoid formation.

Visible Contamination of the Monochromatic Beam

Although the monochromatic beam of λ2804 Å which emerged from the second monochrometer was of an unusually high degree of purity, the dark-adapted human eye could detect a slight content of bluish or violet visible light. This was presumably the result of a small amount of scattering within the prisms, and it was perhaps even more due to fluorescence of the quartz prisms.[2] Since no information exists as to the minimum dosage of visible light which will affect a population of *Fucus* eggs, a collateral series of experiments was designed to show conclusively whether the observed results are attributable to the ultraviolet and not to the minute amount of visible light. Two plates of clear glass each 6.4 mm. thick were interposed to absorb the ultraviolet while permitting the visible (as well as long ultraviolet, if any) to pass. The radiation which passed through the glass was

[2] The wave-length 2537 Å caused a considerably more marked fluorescence of the fused quartz prism.

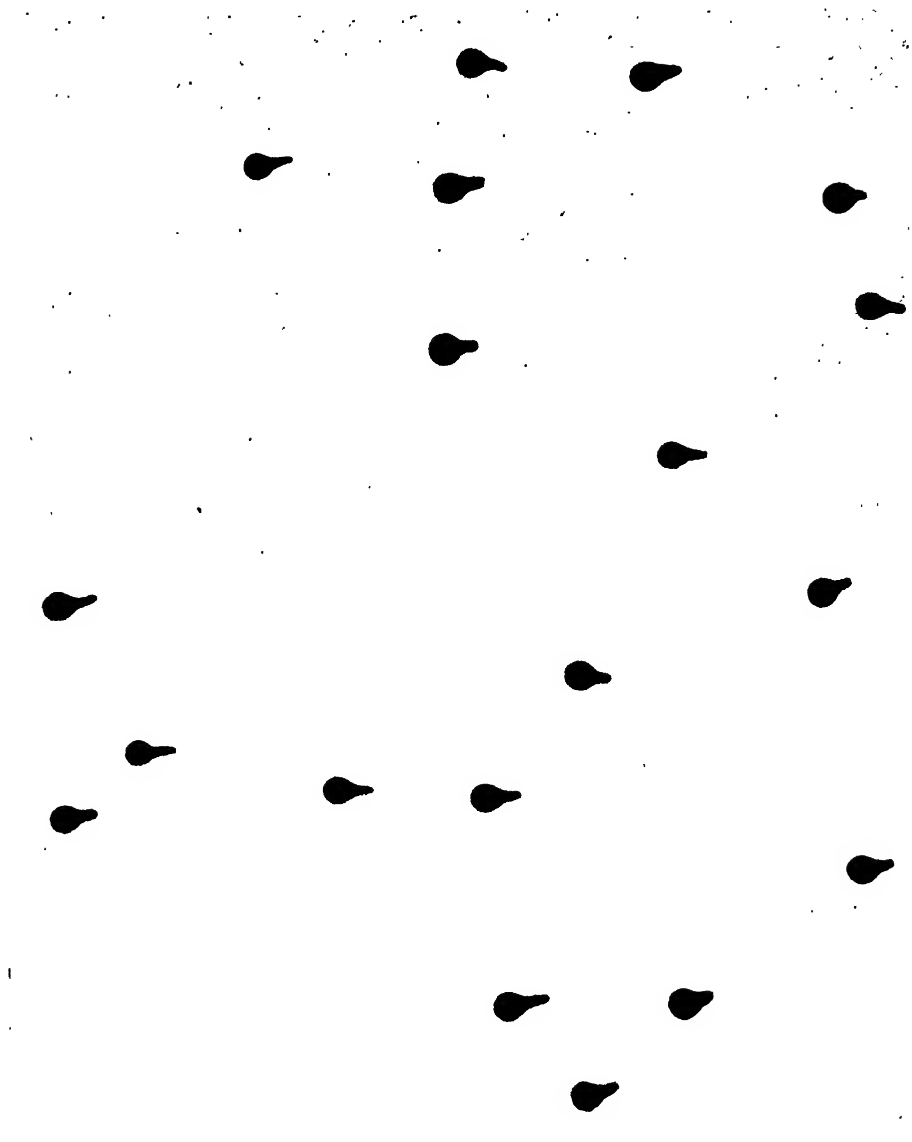

FIG. 2. Photomicrograph of a small area of the bottom of a quartz culture vessel after rhizoids have formed. The culture was irradiated (λ2804 Å) unilaterally from the left hand side during the period 7–9 hours after fertilization with 10,000 units (1 unit representing 1.2 ergs per mm.[2] applied to the side of the culture (see text)). The rhizoids have formed on the halves of the eggs away from the source of radiation.

tested on seven populations of *Fucus* eggs at 7–8 hours after fertilization. The dosage applied would have been 1000 units were it not for the inter-

ception of ultraviolet by the glass. In these seven experiments 52.3 per cent of 652 eggs formed rhizoids away from the light. Since six of the seven dishes showed a slight increase above 50 per cent, a small response appears to exist. An inspection of curve *a*, Fig. 1, shows that if the glass had not been present, 1000 units would have caused 93 per cent of the rhizoids to form away from the light. If curve *a*, Fig. 1, is extrapolated to 52.3 per cent, it is found that this response would be expected from a dosage of 2 units, which is 0.2 per cent of 1000 units. It is clear therefore that almost all of the response in the experiments represented by curve *a*, Fig. 1, is due to ultraviolet light. The small response when the glass is interposed may be due to the visible light, but even it is probably due more to ultraviolet since nearly 0.2 per cent of λ2804 Å may reasonably be expected to pass through the glass.

Fluorescence of the Vessels

During irradiation with λ2804 Å, the walls of the quartz culture vessels emit a very dim visible light as a result of fluorescence. To test the effect of this fluorescent light four culture vessels containing populations of eggs which had been fertilized 7 hours were placed beside similar vessels and eggs which were directly in the ultraviolet beam. The vessels not in the beam were separated from those in the beam by 6.4 mm. of plate glass to stop any reflected ultraviolet while permitting the visible fluorescent light to pass. The vessels in the beam were irradiated with 10,000 units over a period of 2 hours. The eggs in these vessels responded as in curve *a*, Fig. 1 (98 per cent). Of 257 eggs which were subjected only to the fluorescent light coming from one side, 50.2 per cent formed rhizoids away from the light. Two of the populations formed slightly more and two slightly less than 50 per cent away. The dim fluorescent light from the walls of the vessels is thus seen to be ineffective.

The Second Series of Experiments

A second series of experiments was carried out, principally in February, in which a constant dosage (5000 units) was applied at a variable time after fertilization. Throughout the whole series the variation in intensity of the beam was such that the time required to apply 5000 units ranged from 45 to 62 minutes. With a few exceptions, the irradiating was started exactly at the beginning of each hour from 3 to 10 hours after fertilization. The results are shown in Fig. 3, in which the points are located on the time (horizontal) axis at the midpoint of the period of irradiation. Each point represents the average of the results of five to seven experiments involving a total of 410 to 665 eggs, except that the last two points are based on four

experiments. It is seen in Fig. 3 that the response of the eggs is not great until some time after fertilization. It does not become maximal until the interval 7–8 hours after fertilization, but it remains maximal for some time thereafter. In these respects, and in general, the increase in response with age resembles the response to white light (Whitaker and Lowrance, 1936). Eggs were not irradiated earlier than 3 hours after fertilization because they do not become firmly attached to the bottom of the dish until nearly this time.

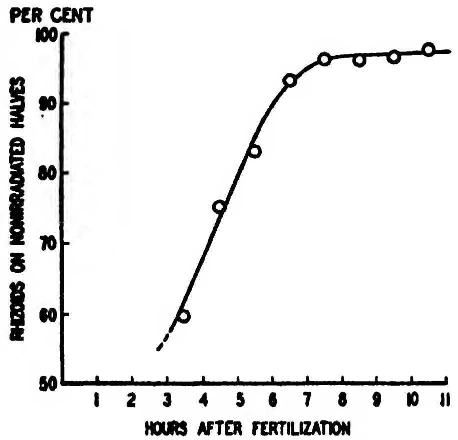

FIG. 3. The vertical axis indicates the percentage of eggs which formed rhizoids on the halves away from the source of radiation (λ2804 Å) after unilateral irradiation. The horizontal axis indicates the time after fertilization when a dosage of 5000 units was applied, 1 unit representing the application of 1.2 ergs per mm.^{2} to the side of the culture (see text). Dosage was applied during a period of 45–62 minutes, and the points on the curve are placed on the time axis at the midpoint of the period of exposure. Each point represents the average of four to seven experiments involving a total of 410 to 665 eggs.

The Third Series of Experiments

In March, in the course of some other experiments, it was found that the responsiveness of the eggs had apparently increased. The *Fucus* had also come into full ripeness. Gametes were shed copiously, in contrast to the condition in January, and the vegetative spring growth of the plants had begun. In the January experiments (curve *a*, Fig. 1) the six quartz culture vessels had been used at random, without mark or distinction, and either polished side had faced the beam. By March one of the six vessels had been broken and the remainder had been numbered. It had been found that some of the vessels consistently gave higher percentages than others, and that the two polished sides of some of the vessels also differed significantly. In view of the apparent increase in sensitivity of the eggs, a third series of experiments, in general similar to the first, was carried out in March to test again the logarithmic relation of response and dosage which was found in the first series. An equal number of experiments with each vessel was carried out at each dosage so that differences in the vessels would cancel out with equal weighting in the averages. The same side of each

vessel faced the beam. Twenty experiments, in the course of which each vessel was used four times, were carried out at each of the following dosages: 1, 5, 10, 100 units. At each dosage 1529 to 2200 eggs were counted. In all cases the eggs were irradiated within the period 8–9 hours after fertilization, and the duration of the irradiation was approximately as follows: 1 unit, 0.7–0.8 seconds; 5 units, 3.2–3.6 seconds; 10 units, 5.8–7.0 seconds; 100 units, 60–71 seconds.

The results are shown in Fig. 1, curve *b*. It is seen that the eggs responded with greater sensitivity than they did in January (curve *a*). Since the quartz vessels were used differently, it is not certain how much of this increased sensitivity is due to a difference in the eggs and how much is only apparent, due to the eggs having actually received a greater amount of ultraviolet per unit applied to the vessel. A somewhat greater number of eggs was placed in each vessel in the March series, due to improved technique, but the population density was quite uniform throughout the series. The greater number of eggs would tend to increase the eclipsing and reduce the average amount of energy received by the eggs. Curve *b*, Fig. 1, is very nearly parallel to curve *a*, and it is also essentially a straight line over the range covered, confirming the logarithmic relation of dosage and response.

Other Wave-Lengths

Four shorter wave-lengths, 2654, 2537, 2482, and 2345 Å, were tested in an exploratory way in a number of similar experiments to see if they also could determine the place of rhizoid origin. All of these wave-lengths were highly effective, and in sufficient dosage caused 100 per cent of the rhizoids in some of the vessels to form away from the source of radiation. While the results are inadequate to permit an exact comparison of the effectiveness of these wave-lengths with each other and with λ2804 Å, it can safely be said that their effectiveness is of the same order of magnitude. Some of them are probably more effective than λ2804 Å.

Two longer wave-lengths were also tested: λ3130 and 3660 Å. These were effective, but only when more total energy was applied. A great increase of total energy was necessary to give high percentage response. Very much larger doses were also received by the eggs without inhibition of rhizoid formation. 96 per cent of the eggs formed rhizoids away from the source of radiation when 577,000 units of λ3130 Å were applied; and 1,000,000 units of λ3660 Å caused 100 per cent of the eggs to respond in a similar manner. These large dosages were applied at a rapid rate. It may be recalled that 20,000 units of λ2804 Å inhibited rhizoid formation.

Absorption

Five measurements were made of the extent to which a beam of λ2804 Å is extinguished in passing through a single layer of *Fucus* eggs. Fertilized eggs were packed tightly on the bottom of a cylindrical depression in a quartz slide by means of a lip pipette. Unusually large and small eggs were discarded. The average diameter of the eggs in these measurements was 76 μ. The slide had been painted black except over the bottom of the depression containing the eggs. The intensity of the beam from a constant source was measured after passing through the slide with sea water in the depression, and again when a single layer of packed eggs was on the bottom of the depression. In the first two measurements, one on eggs fertilized 2 hours and the other on eggs fertilized 6 hours, the total area of the eggs was estimated by calculating the average projected area of the eggs from measurements of diameter, and counting the total number of eggs in the depression (approximately 3800). From the intensity measurements and the percentage of the total area covered by eggs it was calculated that in both cases 100 per cent of the radiation incident on the eggs was extinguished. In three other measurements eggs were used which had been fertilized 3, 3½, and 6½ hours and another method of calculation was employed. Perfectly packed spheres of the same diameter cover in projection 90.7 per cent of the total area. Assuming that *Fucus* eggs do so, it was calculated from the intensity measurements, with and without eggs in the depression, that 96, 95, and 97 per cent of the radiation incident on the eggs was extinguished in the three cases. Actually the geometric fit of the eggs was not perfect and the total space between eggs was probably somewhat more than assumed. If so, the percentage extinction would be slightly greater than calculated. It therefore appears that very nearly if not all of the beam incident on the eggs is extinguished whether the eggs have been fertilized 2, 3, 3½, 6, or 6½ hours. Extinction results from the combined effects of absorption and scatter.

Three similar measurements of extinction of a beam of λ3660 Å indicated 86, 84, and 87 per cent extinction. The second method of calculation mentioned above was used, and the eggs had been fertilized 7, 3½, and 6½ hours. The extinction of λ3660 Å is thus definitely less than the extinction of λ2804 Å. Although it is not known what part of the extinction is due to absorption and what part to scattering, it appears that λ3660 Å is less absorbed than λ2804 Å. However, the difference in proportion of energy absorbed by the eggs in the two cases is not nearly so great as the difference in effectiveness of the two wave-lengths. While 100 units of λ2804 Å

caused more than 90 per cent of the eggs to form rhizoids away from the source of radiation, 50,000 units of λ3660 Å caused slightly less than 90 per cent to do so. 20,000 units of λ2804 Å inhibited rhizoid formation, while 1,000,000 units of λ3660 Å did not. It is quite possible that λ2804 Å and shorter wave-lengths are absorbed largely in the cortex of the egg so that the effect may be very concentrated locally, and conducive to a sharp gradient.

DISCUSSION

Heilbrunn (1937) and Heilbrunn and Mazia (1936) review some of the general effects of ultraviolet radiation on protoplasm and cite original sources. It appears probable that cell membrane permeability is locally increased, and that the surface charge, at least of certain bacteria, is decreased. The viscosity of the interior of many cells is increased, after a transitory decrease. The increase begins at the cortex and spreads inward. The viscosity increase is dependent upon calcium, and Heilbrunn (1937) attributes the internal increase of viscosity, as well as other effects of ultraviolet, to a release of protein-bound calcium from the cell surface to the interior.

Some of the effects of ultraviolet light are of a type tending to result from visible light as well, or from radiation in general. Other effects, especially those of a destructive nature which are caused by the middle and shorter ultraviolet, do not result from visible light. Some of these effects may have pronounced physiological consequences without killing the cells. Unlike visible light, ultraviolet activates a number of eggs (*e.g.*, starfish, Lillie and Baskerville, 1922 *a*; sea urchin, Lillie and Baskerville, 1922 *b*), but only if calcium is present (sea urchin, Heilbrunn and Young, 1930). Harvey and Hollaender (1938) have shown that short ultraviolet (λ2260–2480 Å) activates non-nucleated fragments of sea urchin eggs, obviously by action on the cytoplasm. Hollaender (personal communication) has also observed that ultraviolet activates *Fucus* eggs. Ultraviolet induces mutations in *Drosophila* (Altenburg, 1934) and in corn pollen (Stadler and Sprague, 1936). λ3130 Å and longer wave-lengths are relatively ineffective in the case of the corn pollen. Wave-lengths shorter than 3000 Å are absorbed by proteins, which they denature (Clark, 1936), and they also inhibit plant growth (Popp and Brown, 1936).

Since the place of rhizoid origin in the *Fucus* egg may be determined by applying gradients of a considerable number of chemical substances and physical conditions (see Introduction), the fact alone that unilateral irradiation by ultraviolet light is effective gives no clue as to which of the

general or more nearly specific effects of ultraviolet light is involved, or whether it is a combination of effects. Fig. 1 shows that each successive increment of dosage is less effective than its predecessor in evoking further response of a population of eggs. The response is proportional to the logarithm of the total dosage applied. The rhizoid formation itself is of course not a simple process, but this simple relation of dosage to response suggests that the radiation acts on the population in a relatively simple way rather than through a complicated combination of general effects. No conclusion can be drawn at present as to the mode of action of the ultraviolet in the egg, but two reactions in particular are suggestive and perhaps may be profitably considered: The denaturation of protein, and the inactivation of growth substance (auxin).

The action of ultraviolet light on proteins is reviewed by Clark (1936) and is considered in a review of protein coagulation by Anson (1938). Anson states that protein denaturation is a monomolecular reaction, and that denaturation by ultraviolet probably breaks bonds not broken in ordinary heat denaturation. Denaturation is commonly followed by coagulation, but even when it is not the viscosity of a protein solution is increased by denaturation. Clark (1936) cites work which indicates that denaturation causes a certain amount of pH change, acid solutions becoming more basic, and *vice versa*. Localized denaturation on one side of a cell might therefore result in an internal pH gradient. Since *Fucus* eggs do not transmit λ2804 Å readily, such localization is to be expected. It is already known (Whitaker, 1938) that pH gradients can determine the developmental axis. Denaturation would result in physiological and metabolic changes which would obviously be extensive and complicated. Localized denaturation would give rise to gradients across the cell, and in the *Fucus* egg the axis of differentiation may be determined by externally caused gradients of a number of substances and conditions, as postulated by Child (1940) in his generalized concept of the origin of axial differentiation. Full understanding of the nature of the differentiation processes must ultimately depend on discovering their specific chemical basis.

If the response of *Fucus* eggs to shorter ultraviolet results from localized protein denaturation, the effectiveness of different wave-lengths shorter than 2900 Å should correlate with the typical absorption curve of proteins. As indicated earlier, the exploratory measurements made at wave-lengths shorter than 2804 Å are not adequate to show whether this is the case, and this question remains to be answered.

Plant growth is inhibited by short ultraviolet, and one of the ways in which it exerts its effect appears to be to inactivate or destroy growth

hormone (auxin). Went and Thimann (1937) in their monograph on phytohormones cite work showing that unfiltered ultraviolet inactivates auxin solutions almost completely. Wave-lengths between 2300 and 3300 Å inactivate auxin-a lactone with great rapidity, and ultraviolet light can inactivate auxin-a by shifting the place of attachment of an OH group. Skoog (1935) has shown that hard x-rays inactivate auxin *in vitro* and *in vivo*.

Growth substance, or auxin, has been extracted from *Fucus* eggs by Du Buy and Olson (1937). Van Overbeek (1940) has recently shown that auxin is present in a number of algae in concentrations comparable to those in higher plants such as corn and pea seedlings. He has further shown in the brown alga *Macrocystis* that beta-indole acetic acid (hetero-auxin), or a substance closely related to it, is present rather than auxin-a or auxin-b which are commonly found in higher plants. The presence of beta-indole acetic acid does not prove its activity in the growth of the plant, but activity in the algae is suggested both by the experiments of Olson and Du Buy (1937) who found that gradients of beta-indole acetic acid can determine the developmental axis of the *Fucus* egg, and by van Overbeek's observations on the differential distribution in *Macrocystis* which suggests hormonal function. The beta-indole acetic acid is most concentrated in young blades. The rôle of auxin in the rhizoid formation in *Fucus* has been recently discussed (Whitaker, 1940 *a*) and for present purposes it need merely be noted that inactivation or destruction, on one side of the egg, of beta-indole acetic acid, or some auxin-like substance active in rhizoid formation, might be an important factor in the present instance. If the destruction extended throughout the entire egg, rhizoid inhibition would be expected and this is observed after strong dosages, without cytolysis or visible breakdown of the protoplasm.

The shorter wave-lengths of the visible spectrum also cause *Fucus* eggs to respond. Whether they act in the same way as ultraviolet of λ2804, or less, Ångstrom units cannot be decided at present. It is not known whether the response of a population to visible light follows the logarithm of the dosage. Shorter visible wave-lengths are known to affect the transport and activity of auxin in higher plants. The fact that λ2804 Å, which has destructive powers not possessed by the long ultraviolet (λ3660 Å), is much more effective than λ3660 Å suggests that destructive effects are involved in the action of λ2804 Å.

The eggs do not become fully responsive to ultraviolet light until about 7 hours after fertilization, as may be seen in Fig. 3. One explanation of this might be that some substance acted upon by the ultraviolet gradually forms

after fertilization and is not present in maximum concentration until after 7 hours have passed. A more probable interpretation is that the eggs recover from the effect of irradiation after a number of hours, and that at 7 or more hours after fertilization there is not time for recovery before the rhizoids form. The rhizoids begin to form in a population at about 12 hours after fertilization, with the mode at 16–17 hours (Whitaker and Lowrance, 1936). The formation of rhizoids in a population follows the form of a somewhat skewed probability curve so that on the basis of the recovery hypothesis Fig. 3 would be expected to be a sigmoid curve. A similar relation was found in the case of white light (Whitaker and Lowrance, 1936), although the eggs did not respond until somewhat longer after fertilization, which suggests that they take somewhat longer to recover from the effects of λ2804 Å, although less energy was applied.

SUMMARY AND CONCLUSIONS

1. When *Fucus* eggs which have been fertilized for a sufficient length of time are irradiated unilaterally with monochromatic ultraviolet light (λ2804 Å) of adequate dosage, 97–100 per cent form rhizoids on the halves of the eggs away from the source of radiation (see Figs. 1 and 2).

2. The responsiveness of the eggs increases gradually after fertilization and does not reach a maximum until about 7 hours at 15°C. (see Fig. 3). The first rhizoids begin to form in a population at about 12 hours after fertilization. The responsiveness remains maximal until at least 11 hours after fertilization.

3. It is suggested that the low responsiveness of a population of eggs at an earlier period is due to recovery from the effects of irradiation before the rhizoids begin to form.

4. The response of eggs to λ2804 Å is proportional, over a wide range, to the logarithm of the dosage (see Fig. 1). Dosage was regulated by the duration of exposure during the period of maximum response.

5. High dosages of λ2804 Å, of the order of 10,000 ergs per mm.^{2}, cause the rhizoids to form fairly precisely away from the source of radiation (see Fig. 2). Twice this dosage inhibits rhizoid formation altogether without causing cytolysis.

6. Other wave-lengths which have also been shown to be effective are: 3660, 3130, 2654, 2537, 2482, and 2345 Å. Only exploratory measurements have been made to test the effectiveness of these wave-lengths, but they show that much greater energy is necessary to obtain a strong response with λ3130 and 3660 Å, especially the latter. The wave-lengths shorter than 2804 Å, on the other hand, show the same order of effectiveness as λ2804 Å. Some may be more effective.

7. A beam of λ2804 Å which is incident on a single layer of *Fucus* eggs is completely extinguished at 2, 3, 6, or 6½ hours after fertilization. About 85 per cent of a beam of λ3660 Å is extinguished. The wave-length 3660 Å is thus not so completely absorbed as λ2804 Å, but the difference in proportion absorbed by the egg is not nearly so great as the difference in effectiveness.

The author is indebted to Mr. Emerson Reed for assistance in carrying out the experiments.

BIBLIOGRAPHY

Altenburg, E., 1934, The artificial production of mutations by ultraviolet light, *Am. Nat.*, **68**, 491.

Anson, M. L., 1938, Certain chemical and physical characteristics of the proteins. Section I. The coagulation of proteins, in Schmidt, C. L. A., The chemistry of the amino acids and proteins, Springfield, Illinois, Charles C. Thomas, 407.

Child, C. M., 1940, Lithium and echinoderm exogastrulation—with a review of the physiological gradient concept, *Physiol. Zool.*, **13**, 4.

Clark, J. H., 1936, The effect of radiation on proteins, in Duggar, B. M., Biological effects of radiation, New York, McGraw-Hill.

Du Buy, H. G., and Olson, R. A., 1937, The presence of growth regulators during the early development of *Fucus*, *Am. J. Bot.*, **24**, 609.

Gardner, N. L., 1922, The genus *Fucus* on the Pacific coast of North America, *Univ. Calif. Pub. Bot.*, **10**, No. 1, 1.

Harvey, E. B., and Hollaender, A., 1938, Parthenogenetic development of the eggs and egg fractions of *Arbacia punctulata* caused by monochromatic ultraviolet radiation, *Biol. Bull.*, **75**, 258.

Heilbrunn, L. V., 1937, An outline of general physiology, Philadelphia, W. B. Saunders.

Heilbrunn, L. V., and Mazia, D., 1936, The action of radiation on living protoplasm, in Duggar, B.M., Biological effects of radiation, New York, McGraw-Hill.

Heilbrunn, L. V., and Young, R. A., 1930, The action of ultraviolet rays on *Arbacia* egg protoplasm, *Physiol. Zool.*, **3**, 330.

Hurd, A. M., 1920, Effect of unilateral monochromatic light and group orientation on the polarity of germinating *Fucus* spores, *Bot. Gaz.*, **70**, 25.

Knapp, E., 1931, Entwicklungsphysiologische Untersuchungen an Fucaceen-Eiern. I. Zur Kenntnis der Polarität der Eier von *Cystosira barbata*, *Planta*, **14**, 731.

Kniep, H., 1907, Beiträge zur Keimungs-Physiologie und Biologie von *Fucus*. *Jahrb. wissensch. Bot.*, **44**, 635.

Lillie, R. S., and Baskerville, M. L., 1922 *a*, The action of ultraviolet rays on starfish eggs, *Am. J. Physiol.*, **61**, 57.

Lillie, R. S., and Baskerville, M. L., 1922 *b*, The action of ultraviolet rays on *Arbacia* eggs, especially as affecting the response to hypertonic sea water, *Am. J. Physiol.*, **61**, 272.

Lowrance, E. W., 1937, Effect of temperature gradients upon polarity in eggs of *Fucus furcatus*, *J. Cell. and Comp. Physiol.*, **10**, 321.

Lund, E. J., 1923, Electrical control of organic polarity in the egg of *Fucus*, *Bot. Gaz.*, **76**, 288.

Olson, R. A., and Du Buy, H. G., 1937, The rôle of growth substance in the polarity and morphogenesis of *Fucus*, *Am. J. Bot.*, **24**, 611.

Popp, H. W., and Brown, F., 1936, The effect of ultraviolet radiation on seed plants, in Duggar, B. M., Biological effects of radiation, New York, McGraw-Hill.

Skoog, F., 1935, The effect of X-irradiation on auxin and plant growth, *J. Cell. and Comp. Physiol.*, **7**, 227.

Stadler, L. J., and Sprague, G. F., 1936, Genetic effects of ultraviolet radiation in maize. II. Filtered radiations, *Proc. Nat. Acad. Sc.*, **22**, 579.

Van Overbeek, J., 1940, Auxin in marine algae, *Plant. Physiol.*, **15**, 291.

Went, F. W., and Thimann, K. V., 1937, Phytohormones, New York, Macmillan Co.

Whitaker, D. M., 1931, Some observations on the eggs of *Fucus* and upon their mutual influence in the determination of the developmental axis, *Biol. Bull.*, **61**, 294.

Whitaker, D. M., 1936, The effect of white light upon the rate of development of the rhizoid protuberance and the first cell division in *Fucus furcatus*, *Biol. Bull.*, **70**, 100.

Whitaker, D. M., 1937 *a*, The effect of hydrogen ion concentration upon the induction of polarity in *Fucus* eggs. I. Increased hydrogen ion concentration and the intensity of mutual inductions by neighboring eggs of *Fucus furcatus*, *J. Gen. Physiol.*, **20**, 491.

Whitaker, D. M., 1937 *b*, The effect of hydrogen ion concentration upon the induction of polarity in *Fucus* eggs. II. The effect of diffusion gradients brought about by eggs in capillary tubes, *J. Gen. Physiol.*, **21**, 57.

Whitaker, D. M., 1937 *c*, Determination of polarity by centrifuging eggs of *Fucus furcatus*, *Biol. Bull.*, **73**, 249.

Whitaker, D. M., 1938, The effect of hydrogen ion concentration upon the induction of polarity in *Fucus* eggs. III. Gradients of hydrogen ion concentration, *J. Gen. Physiol.*, **21**, 833.

Whitaker, D. M., 1940 *a*, The effects of ultra-centrifuging and of pH on the development of *Fucus* eggs, *J. Cell. and Comp. Physiol.*, **15**, 173.

Whitaker, D. M., 1940 *b*, The effect of shape on the developmental axis of the *Fucus* egg, *Biol. Bull.*, **78**, 111.

Whitaker, D. M., and Lowrance, E. W., 1936, On the period of susceptibility in the egg of *Fucus furcatus* when polarity is induced by brief exposure to directed white light, *J. Cell. and Comp. Physiol.*, **7**, 417.

Whitaker, D. M., and Lowrance, E. W., 1940, The effect of alkalinity upon mutual influences determining the developmental axis in *Fucus* eggs, *Biol. Bull.*, **78**, 407.

Winkler, H., 1900, Ueber den Einfluss äusserer Factoren auf die Theilung der Eier von *Cystosira barbata*, *Ber. bot. Ges.*, **18**, 297.

ON THE RELATION BETWEEN GROWTH AND RESPIRATION IN THE AVENA COLEOPTILE

By BARRY COMMONER AND KENNETH V. THIMANN

(*From the Biological Laboratories, Harvard University, Cambridge*)

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I

INTRODUCTION

The discovery of the auxins and their activity in controlling plant growth has raised certain questions of a novel type. Of these perhaps the most fundamental is that of the mechanism of the action: namely, of how a single substance can bring about the elaborate series of changes that comprise growth. Attempts at a solution of this problem have been made along the following three lines: (1) study of the chemical nature of the auxins; (2) study of the mechanical changes, particularly those of the cell wall, which accompany growth; and (3) analysis of the intermediate stages between the supplying of the hormone and the first appearance of the growth phenomena. The results of these experiments have been discussed by Went and Thimann (1937) and by Thimann and Bonner (1938) and need not be considered as a whole here. The essential data pertinent to the present research are as follows:

A close connection exists between growth and respiration. At least in the *Avena* coleoptile, growth will not take place without respiration; and if respiration is partially reduced by treatment with the proper concentration of cyanide, growth is reduced to the same extent (Bonner, 1936).

A close connection also exists between growth and protoplasmic streaming. Those concentrations of auxin which accelerate growth also accelerate the rate of streaming, and the effect on streaming is observable long before any effect on growth can be detected (Thimann and Sweeney, 1937). For both growth and streaming sugar plays an essential rôle as an accessory substance; it increases the amount of growth resulting from a given auxin concentration (Schneider, 1938), and it prolongs the acceleration of streaming caused by auxin (Sweeney and Thimann, 1938).

The effect of various respiratory substrates on growth also serves to indicate an interrelation between growth and respiration. Thus, sugar is essential to the growth of isolated coleoptile sections, and the four-carbon

acids, malic and fumaric, when supplied together with auxin and sugar further increase the growth of such sections. This latter finding, which was observed in certain preliminary experiments, served as a guide to the work to be discussed, and it will be shown that this effect sheds a good deal of light on the relation between auxin and respiration.

All these data point in the direction of close interrelation between the processes of growth and respiration. Several attempts have been made to demonstrate some respiratory effect of auxin. However, in all cases the addition of auxin (in concentrations which accelerate growth) had no effect on the rate of respiration of *Avena* coleoptiles (Bonner, 1936;[1] Van Hulssen, 1934). It was apparent therefore, that the problem called for an examination in greater detail.

The present paper represents a study of the relation between growth and respiration in the *Avena* coleoptile, and an attempt to analyze the physiological basis of the effects of auxin.

II

Materials and Methods

Avena coleoptiles were grown in the usual way on filter paper in a dark room at 24°C. and 85 per cent relative humidity, with occasional red light. The plants were used at the age of 76 hours from the time of soaking unless otherwise stated.

As auxin, pure indole-3-acetic acid was used throughout. The malic acid used was decolorized and recrystallized from a C.P. sample. All solutions were made up freshly once a week. Acid solutions were neutralized to pH 6.8 with KOH.

Measurements of growth were made on isolated sections of the coleoptile 3 mm. long placed on combs floating on the surface of the solution used (as described by Schneider, 1938). Thirty sections obtained from ten coleoptiles were used in each such experiment. Respiration was measured either in Warburg manometers using thirty sections or in the microrespirometer previously described by Thimann and Commoner (1940) using only a single section. In some cases the sections used for respiration experiments were measured for length by direct examination with a calibrated microscope (at the end of the run). The growth measurements were carried out in the dark moist room at 24° and the respirometer water baths were held at the same temperature and shielded from light.

III

EXPERIMENTAL

The fact that growth is dependent upon the presence of oxygen and that both growth and respiration are proportionally inhibited by cyanide, provides a clue to the nature of the link between these processes.

[1] In earlier work Bonner (1934) reported that growth substance stimulated the respiration of *Avena* coleoptiles. He later concluded that this effect was due to impurities in the preparation since subsequent measurements with pure crystalline auxin had no effect (1936).

The two end substances involved in respiration are oxygen and the substrate which is oxidized (*e.g.*, sugar). In many plant and animal cells the Warburg-Keilin respiratory system appears to mediate 90 per cent of the oxidation. According to this scheme, molecular oxygen is activated by an oxidase enzyme, and the metabolite is prepared for oxidation by the system of dehydrogenases specific to it. Intermediate carriers link these two ultimate enzymes. Thus:

$$\text{Oxygen} \rightarrow \text{oxidase} \leftrightarrows \text{carriers} \leftrightarrows \text{dehydrogenase} \leftarrow \text{metabolite}$$

Cyanide poisons the (cytochrome) oxidase and thereby prevents the utilization of molecular oxygen. Since the reduction of respiration caused by various concentrations of cyanide is always accompanied by a proportional decrease in growth, it is indicated that the oxidase is necessary for *both* processes. On the other hand we know that respiration may take place without any accompanying growth, so that the same *dehydrogenases* cannot be necessary factors for both processes. It follows that if any separation between growth and respiration is to be experimentally effected, this must occur at the dehydrogenase end of the respiratory sequence.

It was therefore decided to test the effect of various known inhibitors and accelerators of dehydrogenase activity upon the growth rate.

1. The Effect of Dehydrogenase-Active Substances

Growth measurements were made on sections of the *Avena* coleoptile, using the method described above. Combs carrying thirty sections were floated on solutions made up to a concentration of 1 per cent sucrose and 1 mg. per liter of indole-3-acetic acid. To this solution were added various dehydrogenase-active substances and growth measurements made over a period of about 48 hours.

Table I gives the relative growth in 24 hours of sections floated on the various solutions. From this it can be seen that the complete activity of the various dehydrogenases is necessary for complete growth. Urethane, which is a general inhibitor of dehydrogenases, causes a noticeable diminution of growth. Pyrophosphate, which is known to inhibit the succinic dehydrogenase, has a more marked effect. Malonic acid, another inhibitor of succinic dehydrogenase, has a similar effect on growth; while barbital, which is structurally related to malonic acid, exerts a more marked inhibition.

The most striking reduction in growth was obtained with (mono)-iodoacetic acid. This effect which was noticed in passing by Bonner (unpublished) has been recently reported also by Howard and McClintock (1940). Our experiments were carried out independently of those of How-

ard and McClintock but in so far as they are comparable our results do not disagree with those obtained by them.

Iodoacetic acid is known to inhibit a number of dehydrogenases and related enzymes. Among those enzymes affected are glyoxalase, "apozymase," lactic dehydrogenase, malic dehydrogenase, β-hydroxybutyric dehydrogenase, alcohol dehydrogenase, α-glycerophosphate dehydrogenase, and phosphorylating enzymes (*cf.* Cohen, 1939). The effect of iodoacetic acid on these enzymes seems greatly to depend on the concentration of the poison and on the state of the enzyme (*i.e.* whether *in vivo*, *in vitro*, *etc.*) and in some cases is in dispute. However, in the present case, as the data presented below demonstrate, the iodoacetic acid effect seems to be rather specific.

TABLE I

Substance	Concentration	Relative growth in 24 hrs.
Sucrose	1 per cent	100
Auxin	1 mg./l.	
Sucrose alone	1 per cent	54
Iodoacetic acid + sucrose + auxin*	0.01 M	−12
Malonic acid + sucrose + auxin	0.01 M	39
Barbital + sucrose + auxin	0.01 M	7
Urethane + sucrose + auxin	0.01 M	58
Pyrophosphate + sucrose + auxin	0.01 M	33

* These solutions all include 1 per cent sucrose and 1 mg. of auxin per liter.

The effect exerted on growth by various metabolites which would be expected to be dehydrogenated by the coleoptiles is shown in Fig. 1. Sucrose, of course, increases the growth of sections in auxin (Schneider, 1938). In the presence of sucrose and auxin together, succinate, malate, fumarate, and pyruvate all increase the growth rate over that in sucrose and auxin alone. The acceleration of growth by the four-carbon dicarboxylic acids (and pyruvic acid) occurs only after the first day of growth.

It is clear, therefore, that certain inhibitors (iodoacetate in particular) which are known to reduce the respiratory activity of various dehydrogenases, are also inhibitors of growth. Further, the four-carbon acids, and pyruvic acid, which are known to constitute an important reversibly oxidizable chain in the sequential processes of cell respiration (Szent-Györgi, 1935) accelerate the growth rate of coleoptile sections.

2. *The Effect of Iodoacetate on Growth and Respiration*

In the light of the above results it seemed important to examine in greater detail the effect of iodoacetate. Growth measurements were made in the

usual manner, the sections being exposed to solutions containing 1 per cent sucrose, 1 mg. of auxin per liter, and various concentrations of K iodoacetate. The results are shown in Figs. 2 and 3.

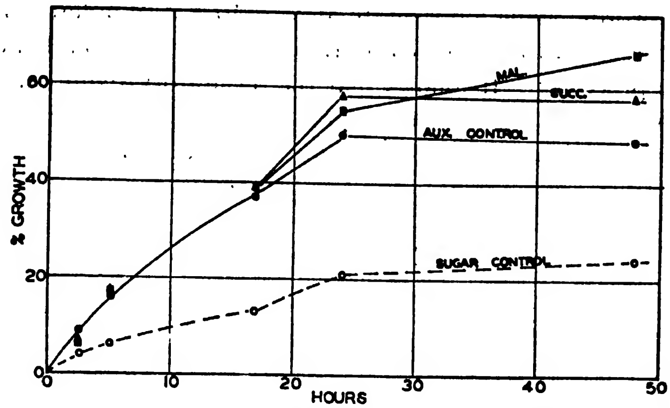

Fig. 1. The effect of malate (0.001 M) and succinate (0.001 M) on the time course of growth. All solutions contained 1 per cent sucrose and all but the sugar control contained 1 mg. of auxin per liter.

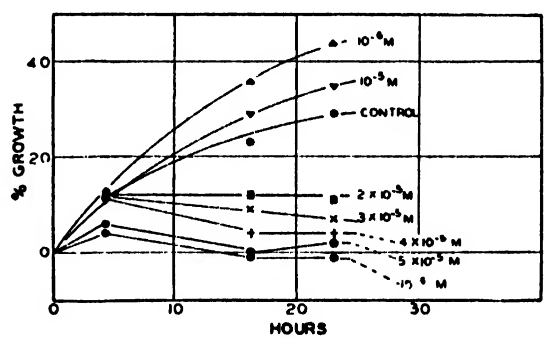

Fig. 2. The effect of various concentrations of iodoacetate on the time course of growth. All solutions contained 1 per cent sucrose and 1 mg. of auxin per liter.

These data demonstrate that the effect of iodoacetate on growth is greatly dependent on concentration. Concentrations of 10^{-6} or 10^{-5} M actually accelerate the growth rate, while concentrations of 2×10^{-5} or greater produce a marked inhibition which becomes complete at 5×10^{-5} M. The shape of the growth curve in Fig. 3 is characteristic of the effect of many poisons and other active substances on enzymatic processes. Thus cyanide and carbon monoxide in low concentrations sometimes accelerate oxygen consumption, and the effect of auxin itself on growth follows a similar curve (Thimann, 1937).

The influence of iodoacetate on the respiration of coleoptile sections was tested over the same range of concentrations. The respiration rates (obtained by Warburg measurements) are plotted in Fig. 3. It is clear

that the effects of iodoacetate on growth and respiration are widely divergent. At a concentration of 5×10^{-5} M, while growth is completely inhibited, the rate of respiration is reduced but 9 per cent. Iodoacetate has no marked inhibitory effect on respiration until a concentration of 10^{-4} M is reached.

In other words, while iodoacetate at 5×10^{-5} M completely blocks the growth processes in the *Avena* coleoptile, it exerts but a small effect on the respiratory processes. It follows that the respiratory requirements of the growth processes cannot represent more than about 10 per cent of the total oxygen consumption, and further that this small fraction of the total

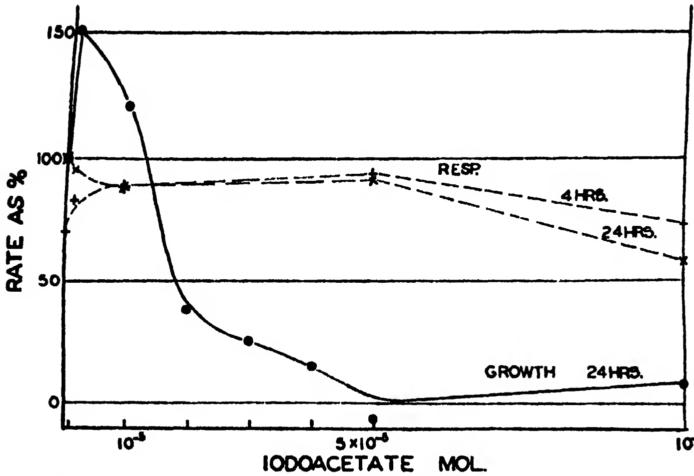

FIG. 3. The effect of iodoacetate on growth and respiration. The growth was measured after 24 hour exposure to solutions containing 1 per cent sucrose, 1 mg. of auxin per liter, and various concentrations of iodoacetate. The respiration rate was determined after 4 hours and after 24 hours.

respiration which is so sensitive to iodoacetate is itself directly concerned with the whole growth process.

3. *The Nature of the Iodoacetate-Sensitive Process*

The above data have shown that iodoacetate poisons a process (or processes) which, while in complete control of growth, involves but a small fraction of the respiration. The next step was to elucidate the nature of this process.

As a first approach the effect of various substances on the inhibition of growth produced by iodoacetate was studied. The substances tested included those which function as coenzymes or substrates in the enzyme processes which are susceptible of iodoacetate inhibition. These were

added to solutions containing 1 per cent sucrose, 1 mg. of auxin per liter, and 5 × 10^{-5} M iodoacetate. Adenine, nicotinic acid, thiamin, and phosphate had no effect on the iodoacetate inhibition. The positive results obtained are shown in Fig. 4. It is clear that of the variety of substances tested, the four-carbon dicarboxylic acids, and pyruvic acid,[2] are alone able to counteract the inhibition induced by iodoacetate. Comparison of this figure with Fig. 1 shows that these substances even produced their usual acceleration of growth as compared with the sucrose-auxin control; *i.e.*, the entire effect of iodoacetate was nullified.

This result enables us to make at least a tentative identification of the iodoacetate-sensitive process. Since the work of Szent-Györgyi (1935)

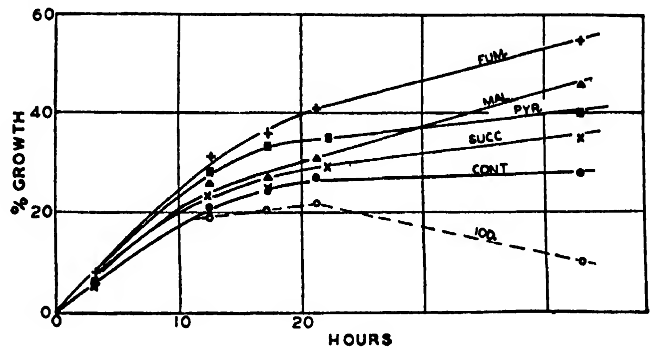

FIG. 4. The effect of four-carbon acids and pyruvate (concentrations: 0.001 M) on the iodoacetate inhibition of growth. All solutions contained 1 per cent sucrose and 1 mg. of auxin per liter. All solutions but the control contained 2 × 10^{-5} M iodoacetate.

it has been known that the four-carbon dicarboxylic acids function as respiratory carriers in most cells. This occurs by way of a series of reversible oxido-reduction reactions:

$$\text{Succinic} \leftrightarrows \text{fumaric} \leftrightarrows \text{malic} \leftrightarrows \text{oxaloacetic}^{3}$$

This sequence serves, in part, to carry the oxidative activity between the cytochrome oxidase and the dehydrogenases. As indicated above, two of the enzymes which mediate these oxido-reduction reactions, malic and succinic dehydrogenases, are inhibited (at least *in vitro*) by iodoacetate. It seems likely therefore that the iodoacetate-sensitive process in the *Avena* coleoptile is the series of four-carbon acid reactions.

[2] The fact that pyruvate behaves in the same way as the four-carbon acids is doubtless due to its participation in the four-carbon acid respiration cycle (Krebs and Eggleston, 1940).

4. The Effect of the Four-Carbon Acids on Respiration

Now, if the above deduction is correct it should be confirmed by an examination of the respiratory activity of the four-carbon acids. It should be possible to show that the coleoptile can oxidize these compounds, and most important of all, that this respiration is in some way controlled by auxin. The experiments which now follow are sufficient, it is believed, to establish these points.

Coleoptile sections which had been soaked overnight in solutions of 1 per cent sucrose plus 1 mg. per liter of auxin, or in sucrose alone, were treated

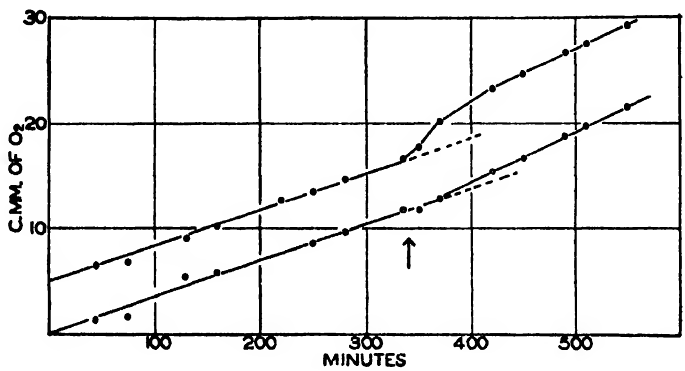

FIG. 5. The influence of auxin on the respiratory effect of malate. The upper curve (closed circles) is for sections which were soaked overnight in 1 per cent sucrose plus 1 mg. of auxin per liter. The lower curve (open circles) is for sections which were soaked overnight in 1 per cent sucrose only. Malate (0.001 M) was added at the arrow mark. The origins are arbitrary.

with 0.001 M malate and the effect on the respiration determined. One set of sections was also soaked in a solution containing sucrose, auxin, and the malate. The respiratory rate of this set was determined on the 2nd day and at the same time the effect of adding malate to the sets soaked in sugar alone and in sugar *plus* auxin was measured. The data presented in Fig. 5 show that when 0.001 M malate is added to the sections, the respiration of those in sucrose alone is increased very slightly, while the sections in sucrose *plus* auxin exhibit a more marked rise in rate.

The presence of malate prevents the fall in respiratory rate which normally occurs during the 1st day after cutting. This decrease in Q_{O_2}[3] can be recovered by adding malate on the 2nd day, but the acceleration of

[3] Q_{O_2} as used in this paper, refers to the number of cubic millimeters of oxygen consumed by thirty coleoptile sections during 1 hour.

respiration caused by the malate is much larger when auxin is present than in sucrose alone.

Hence it is apparent that the respiratory activity of malate is augmented by the presence of auxin. This point will be further developed in the sections below.

5. *The Respiratory Effect of Auxin*

It is now possible to return to the original problem which was the concern of the earlier workers; namely, what is the effect of auxin on the respiration of the *Avena* coleoptile?

The work of Bonner (1936) and Van Hulssen (1934) showed that growth-accelerating concentrations of auxin have no effect on the respiration of the coleoptiles. All of their determinations were made on large numbers of coleoptiles in water or in a sugar solution. However, the conditions of these experiments did not preclude the possibility that auxin might cause a small and transitory change in Q_{O_2}, which could well be masked by the large number of coleoptiles in the respirometer. In fact, the smallness of the fraction of the total respiration which is related to growth lent weight to this suggestion.

Hence these findings were checked by respiration measurements on a single 3 mm. section of a coleoptile in the sensitive microvolumetric respirometer described by Thimann and Commoner (1940). A single section was placed in the respirometer immediately after cutting and floated on 0.1 ml. of distilled water, 1 per cent sucrose, or 1 per cent fructose. After a stable respiration rate had been reached the apparatus was tipped and the section dropped into a solution identical with the original but containing auxin as well. Fig. 6 A which is one of a number obtained in the same way shows that no significant change in the respiration rate can be observed, thus agreeing with the previous work on larger masses of tissue.[4]

However, the importance of the four-carbon acids, made clear above, suggested that the respiratory effect of auxin (if any) might be dependent on the presence of these substances. Consequently, the above experiment was repeated by adding auxin to a section which had been previously soaked for several hours in a solution containing 1 per cent sucrose and 0.001 M K malate. Fig. 6 B shows that an increase in Q_{O_2} of about 14 per cent results from the addition of auxin in a concentration of 1 mg. per liter.

[4] Du Buy and Olson (1940), using another method, also found little change in respiration when 1 mg. per liter indole-acetic acid and fructose was added to freshly cut coleoptiles. Their published curve appears, however, to show a slight increase (*cf.* also Fig. 7).

The rise in respiratory rate occurs almost immediately and is noticeable about 15 minutes after the addition takes place.

Since this effect appeared to be stable over a period of several hours, it was possible to investigate it more fully using larger masses of tissue and Warburg respirometers.

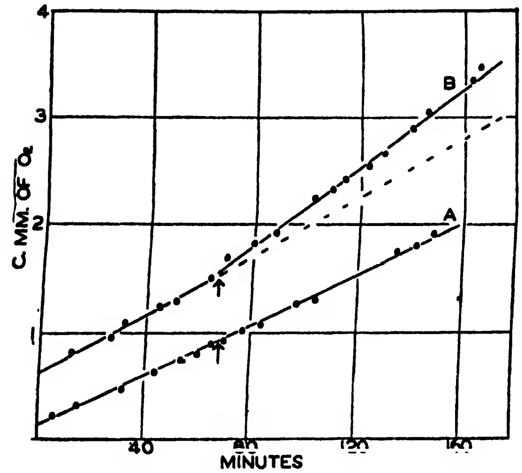

FIG. 6. The effect of auxin on the respiration of freshly cut apical sections of the *Avena* coleoptile. Curve A is for a single section placed in 1 per cent sucrose immediately after cutting, curve B for a single section in 1 per cent sucrose plus 0.001 M malate. Auxin (1 mg. per liter) was added at the point marked by the arrow. It should be noted that these Q_{O_2}'s are high as compared with later measurements made with larger masses of tissue. This is due to the fact that the apical sections have the highest normal Q_{O_2} and that later measurements were made on sections obtained from 9 mm. (subapical) of the coleoptile. The latter therefore have a lower average respiration. The present measurements were made immediately after cutting so that the fall in Q_{O_2} which occurs on standing was avoided. The origins of the curves are arbitrary.

Thirty 3 mm. sections of *Avena* coleoptiles were introduced into the main compartment of the Warburg vessel together with 1.5 ml. of one of the following media: distilled water, 1 per cent sucrose, 1 per cent sucrose plus 0.001 M malate, 1 per cent sucrose plus 0.001 M fumarate. Into the side-arms were introduced 0.5 ml. portions of a solution of auxin in the appropriate medium. The auxin concentration was 4 mg. per liter, thus making 1 mg. per liter when added to the main part of the vessel. In the case of the water experiments, two side-arms were used, one containing a solution of malate (to make 0.001 M when added) and the other a solution of auxin (to make 1 mg. per liter when added).

The sections were soaked in the appropriate medium overnight (*i.e.* the medium to be placed in the main compartment of the vessel). 12 hours after sectioning the respiration rates were measured, and after 4½ hours the side-arm solutions were tipped in. The respiration rate was followed for a number of hours after tipping. The results, presented in Fig. 7, offer clear-cut data on the effect of auxin on the respiration of coleoptile sections:

1. The addition of auxin to sections respiring in water caused no significant change in the rate of respiration (curve A).

2. Sections soaked in sucrose (B, C, D) show an increase in Q_{O_2} of from 8 to 13 per cent (average 10 per cent) on addition of auxin. This increase does not occur until about 1 hour after the addition takes place. In other experiments where the time of soaking in sucrose was less, this increase did not occur at all.

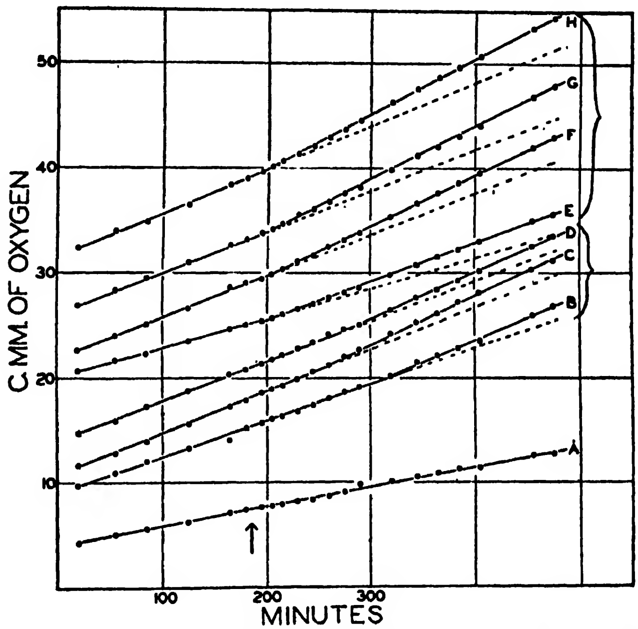

FIG. 7. The effect of auxin on the respiration of sets of thirty coleoptile sections (from ten coleoptiles) under various conditions. A, in water; B, C, D, in 1 per cent sucrose; E, F, G, in 1 per cent sucrose plus 0.001 M malate; H in 1 per cent sucrose plus 0.001 M fumarate. Auxin (1 mg. per liter) was added at the arrow mark. The origin of each curve is arbitrary.

3. On the other hand, the addition of auxin to sections kept in a mixture of sucrose and malate (E, F, G), or in sucrose plus fumarate (H) produced an increase in respiration rate of from 15 to 28 per cent (average 22 per cent).

It was also found that the addition of malate (0.001 M) to water-soaked plants caused no significant change in the rate of respiration.

Now it was shown that the respiratory activity of malate depends on the presence of auxin, and the data of Fig. 7 show that auxin can itself stimulate respiration. This stimulation occurs to a small extent if the

sections have been exposed to sucrose for a long time, but is greatly magnified by the presence of malate or fumarate. There is thus a mutual relationship between the effect of auxin and the four-carbon acids on respiration. If we bear in mind as well the growth experiments of Figs. 1 and 4, it is clear that the four-carbon acid respiration system must be one of the links in the chain of growth processes.

If this is true, the effect of auxin in stimulating growth should parallel closely its effectiveness as an activator of the four-carbon acid respiration.

6. The Identity of the Auxin Effect on Growth and on the Four-Carbon Acid Respiration

The acceleration of growth produced by auxin has long been known to be closely dependent upon the concentration of the hormone. Consequently, the problem of the possible identity of the two auxin effects was studied by determining the variation of their intensities with different concentrations of auxin.

Sets of thirty sections were soaked overnight in a solution of 1 per cent sucrose plus 0.001 M malate and placed in Warburg vessels with 1.5 ml. of the same solution. The side-arms were filled with 0.5 ml. of the mixture made up to contain various auxin concentrations. The effect of adding varying amounts of auxin is shown in Fig. 8 (not all of the concentrations used are shown here). On the 3rd day the sections were removed from the vessels and their lengths measured. The percentage increase in length is plotted in Fig. 9 along with the respiration data of Fig. 8 reduced to percentages.

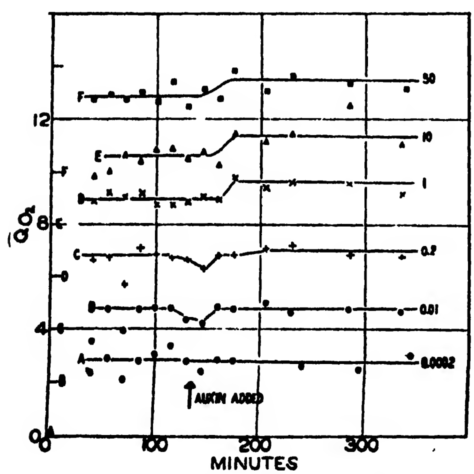

FIG. 8. The effect of various concentrations of auxin on the Q_{O_2} of thirty coleoptile sections. The concentration of auxin in milligrams per liter is indicated on the right of each curve. The zero point for each curve is marked by the position of the letter corresponding to it on the ordinate. For example, the initial Q_{O_2} of curve C is 2.8. All solutions contained 1 per cent sucrose plus 0.001 M malate.

It is clear from this figure that there is a qualitative identity between the effect of auxin on growth and on the four-carbon acid respiration. The curves are decidedly parallel, both showing optima in the range of 1 to 10 mg. of auxin per liter.

7. *Characteristics of the Respiratory Processes Involved in Growth*

In order to study further the properties of the four-carbon acid respiration in the coleoptile it was necessary to deplete the tissues of their reserves of respiratory substrates. This procedure would then reduce the activity of the total respiration, and enable the normally small fraction of four-carbon acid respiration to be measured with greater accuracy. It was shown in Fig. 7 that on soaking the sections in water (or in sucrose solution) the respiratory rate falls off, and that this falling off is prevented by the presence of sucrose and malate together. It may be deduced from

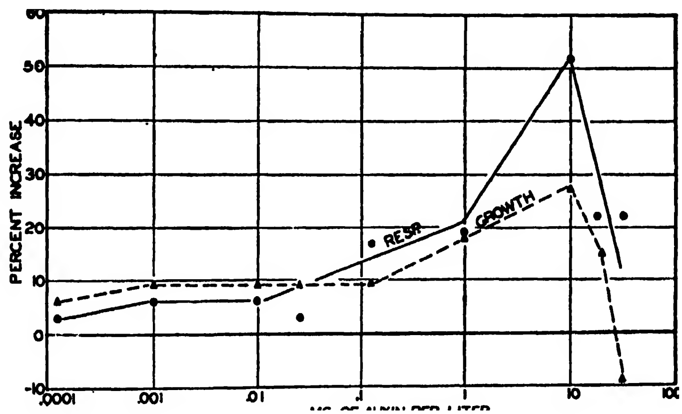

FIG. 9. The effect of various concentrations of auxin on the growth and respiration of coleoptile sections. The data were obtained on the same sets of thirty sections each. The increases noted are relative to the Q_{O_2} and coleoptile length previous to the addition of auxin. All solutions contained 1 per cent sucrose + 0.001 M malate.

this that during soaking in water the reserves of malate (and the other four-carbon acids) are depleted. Hence, if such substances were then added to these sections, the measurement of the resultant respiratory rate could provide an accurate index of the activity of this normally small fraction of the total respiration.

Such experiments were carried out by soaking the sections in distilled water for a period of about 18 hours. At the end of this time the Q_{O_2} (per thirty sections) had fallen to 1.2, less than one-half of the original value. The effect of the four-carbon acids under various conditions was then determined by measuring the Q_{O_2} after the various substances had been added to the starved coleoptile sections. All the solutions used contained 1 per cent sucrose.

The results obtained from this experiment are shown in Fig. 10. Fig.

10*a* shows the effect of various concentrations of malate (in the presence and absence of auxin) on the respiration and growth of the coleoptile sections. If auxin is present, the respiration is markedly dependent upon the concentration of malate, rising to an optimum at 0.001 M and falling off to a low and constant value at higher concentrations. However, if there

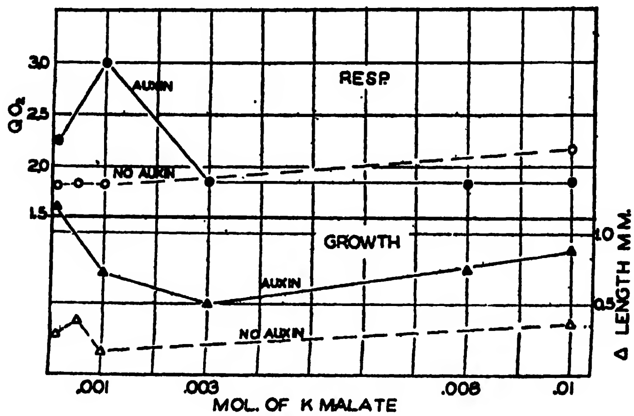

FIG. 10*a*

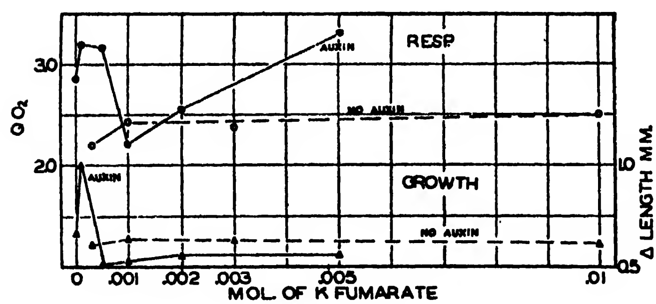

FIG. 10*b*

FIG. 10. The effects of various concentrations of malate (*a*) and fumarate (*b*) on growth and respiration of coleoptile sections. The growth and respiration measurements were made on the same sets of sections in each case. The auxin concentration used was 10 mg. per liter. All solutions used contained 1 per cent sucrose.

is no auxin present, the Q_{O_2} is almost entirely independent of the malate concentration, the rate remaining at the low level characteristic of the supra-optimal concentration of malate in the presence of auxin. Similarly, the length increments,[5] which roughly parallel the Q_{O_2}, are dependent on malate only in the presence of auxin.

[5] It should be pointed out that such starved sections exhibit a smaller response (in growth) to the addition of auxin as compared with freshly cut sections.

The effect of various concentrations of fumarate (in 1 per cent sucrose) on growth and respiration is of a similar nature (Fig. 10*b*). In this case too, the stimulations of growth and respiration parallel each other, at least in the lower concentrations, and occur only if auxin is present.

Thus, when auxin is present, malate and fumarate appear to exert a catalytic effect on the oxidation of the sucrose in the medium. This catalytic effect is indicated by the shapes of the curves, which rise to an optimum Q_{O_2} and then fall off sharply. This suggests that small amounts of these acids can bring about an increase in respiration not by being themselves oxidized, but rather by stimulating the oxidation of some other substrate—such as sucrose. Such an interpretation is of course in keeping with the well established rôle of the four-carbon acids as *carriers* of oxidative processes in the cell.

In the case of fumarate the optimum concentration is somewhat lower than that of malate, and a secondary rise in Q_{O_2} occurs in concentrations greater than 0.001 M. This latter phenomenon seems to indicate that in addition to its catalytic effect on the oxidation of other substrates, fumarate may itself be irreversibly oxidized if present in high concentrations. It is interesting to note, however, that the secondary rise in rate although it is dependent on auxin, does not occur in the effect on growth. Less complete experiments with succinate gave results similar to those obtained with fumarate.[6]

It appears therefore, that in the *Avena* coleoptile the functioning of malate and fumarate as respiratory carriers is dependent on the presence of auxin. Furthermore the catalytic stimulation of respiration by these substances is paralleled by their acceleration of growth. It seems clear that the respiratory activity of the four-carbon acid system is in some manner catalyzed by auxin, and that this activity is one of the requisites for the stimulation of growth by auxin.

IV

DISCUSSION AND CONCLUSIONS

The data here presented provide the basis for a new understanding of the relationship between growth and respiration in the *Avena* coleoptile.

Perhaps the most important conclusion to be drawn is that the effectiveness of auxin as a growth hormone is closely related to its effect on certain respiratory processes; *i.e.*, that auxin itself provides the link between growth and respiration.

[6] As a check on these experiments, a similar run with various concentrations of acetate was carried out. There was no significant effect on the growth or respiration.

When it was first discovered that the growth of plants depended on their respiratory activity, the latter was looked upon as a "primary essential" for growth (Pfeffer, 1900); that is, growth depended on the energy derived from respiration, and so it was logically necessary that respiration be required for growth. This concept of generalized dependence received support, in more recent times, from the finding of Bonner (1936) that cyanide inhibition reduced growth and respiration proportionally.

The present paper indicates that this relationship, rather than being formal, is mediated by certain very specific processes. It has been shown that growth may be completely inhibited by the proper concentration of iodoacetate and the respiratory rate reduced by but 10 per cent. Thus *there must exist some process which, while responsible for but a small part of the normal respiration, is wholly in control of growth and of the effect of auxin on growth.* This finding agrees with the conclusion of Sweeney and Thimann (1938) that auxin accelerates a respiratory process representing only a small part of the total Q_{O_2} but controlling protoplasmic streaming and growth.

It has also been shown that this link is represented by the four-carbon acid respiratory system. This system catalyzes *part* of the total oxidation of respiratory substrates such as sucrose (probably in the form of glucose) but is apparently a direct link in the chain of reactions which is responsible for *all* of the growth.

The four-carbon acid respiration system represents a small but variable fraction of the total Q_{O_2}. In the freshly cut section it accounts for but 10 per cent of the total; and thus, since it alone responds to the addition of auxin, no detectable increase in Q_{O_2} occurs when the hormone is added under these conditions. However, if malate or fumarate is soaked into the coleoptile section, the respiratory *capacity* of this system becomes enlarged, so that when it is activated by addition of auxin a noticeable increase in respiratory rate ensues.

The effect of various concentrations of malate and fumarate indicates that these substances catalyze the oxidation of other respiratory substrates (such as sucrose), although in the case of fumarate, and perhaps succinate, there is evidence to indicate that they may themselves be irreversibly oxidized when present in sufficiently high concentrations. It was not the purpose of the present paper, however, to attempt a detailed analysis of the properties of this respiratory system, but rather to discover the qualitative linkage between growth and the respiratory processes related to it.

The data obtained indicate that the dependence of growth on oxygen consumption is due to the participation of a respiratory process, the four-

carbon acid system, in the chain of growth processes. Further, it is clear that the influence of auxin on growth is related to its effect on this respiratory system.

V

SUMMARY

1. The growth of *Avena* coleoptile sections in sucrose and auxin solutions is inhibited by various substances which are known to act as dehydrogenase inhibitors.

2. Iodoacetate, which is particularly active in this connection, inhibits all growth at a concentration of 5×10^{-5} M, but produces only a slight inhibition of oxygen uptake.

3. The growth inhibition by iodoacetate is completely removed by malate and fumarate, and to a lesser extent by succinate and pyruvate.

4. These acids themselves increase the effect of auxin on growth and also increase the respiration of the coleoptile sections, but only if auxin is present.

5. When sections have been soaked in malate or fumarate, the addition of auxin considerably increases the total respiration. Further, the concentration range over which this increase takes place parallels that active in promoting growth.

6. The four-carbon acids provide a respiratory system which is part of the chain of growth processes, and which is in some way catalyzed by auxin. It represents a small but variable fraction of the total respiration.

REFERENCES

Bonner, J., 1934, *J. Gen. Physiol.*, **17,** 63

Bonner, J., 1936, *J. Gen. Physiol.*, **20,** 1.

Cohen, P. P., 1939, Inhibition of dehydrogenases and related systems, in Elvehjem, C. A., and Wilson, P. W., Respiratory enzymes, Minneapolis, Burgess Publishing Company, 137.

Du Buy, H. G., and Olson, R. A., 1940, *Am. J. Bot.*, **27,** 401.

Howard, F. H., and McClintock, L., 1940, *J. Cell. and Comp. Physiol.*, **15,** 249.

Krebs, H. A., and Eggleston, L. V., 1940, *Biochem. J.*, London, **34,** 442.

Leloir, L. F., and Dixon, M., 1937, *Enzymologia*, **2,** 81.

Pfeffer, W., 1900, The physiology of plants, London, Oxford University Press, translated by A. J. Ewart, 1.

Schneider, C. L., 1938, *Am. J. Bot.*, **25,** 258.

Sweeney, B. M., and Thimann, K. V., 1938, *J. Gen. Physiol.*, **21,** 439.

Szent-Györgyi, A., 1935, *Z. physiol. Chem.*, **236,** 1.

Thimann, K. V., 1937, *Am. J. Bot.*, **24,** 407.

Thimann, K. V., and Bonner, J., 1938, *Physiol. Rev.*, **18**, 524.
Thimann, K. V., and Commoner, B., 1940, *J. Gen. Physiol.*, **23**, 333.
Thimann, K. V., and Sweeney, B. M., 1937, *J. Gen. Physiol.*, **21**, 123.
Van Hulssen, C. J., 1934, Dissertation, University of Utrecht.
Went, F. W., and Thimann, K. V., 1937, Phytohormones, New York, Macmillan Co.

THE EFFECT OF SWELLING ON THE RESPIRATION OF ERYTHROCYTES*

BY F. R. HUNTER AND W. G. BANFIELD

(*From the Department of Zoology, Rhode Island State College, Kingston, Rhode Island*)

(Received for publication, July 22, 1940)

This investigation represents an additional attempt to test the hypothesis that a relationship exists between the respiratory activity of a cell and the maintenance of the selectively permeable properties of its membrane. If this hypothesis were true, it seemed reasonable to expect that a change in the tension of the plasma membrane might possibly bring about a compensatory change in the respiratory activity of the cell. All of the available evidence would indicate that this probably was not the case, but no such assumption can be made *a priori*. A number of investigators have studied the effect of a change in cell size on respiration. Inman (1921 *a*) using the marine alga *Laminaria*, reported a decrease in carbon dioxide production in both hypotonic and hypertonic solutions, while Bodine (1933) using grasshopper eggs in hypertonic solutions and Johnson and Harvey (1938) using marine luminous bacteria in hypotonic solutions, reported a decrease in oxygen consumption. Ray (1927) reported a similar decrease in oxygen consumption of dog reticulocytes in both hyper- and hypotonic solutions. Tipton (1933) found no significant change in the rate of oxygen consumption of chicken erythrocytes using hypotonic and slightly hypertonic solutions of sodium chloride, while Hunter (1939) using the same type of cells in highly hypertonic sodium chloride solutions reported a marked decrease in oxygen consumption. Hypotonic solutions had no effect until a considerable number of cells were hemolyzed. In these solutions there was a marked decrease in oxygen consumption. Ponder (personal communication) found that hypotonic solutions had no effect on the rate of oxygen consumption of rabbit erythrocytes. All of these authors have reported either a decrease in respiration or no change resulting from anisotonic media.

The present investigation was undertaken in an attempt to determine

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what changes in respiration, if any, were associated with the actual swelling of a cell such as an erythrocyte. In the previous investigations, the cells were allowed to reach osmotic equilibrium in the anisotonic medium before respiratory measurements were made. The present data were obtained while the volume changes of the cells were taking place. Chicken erythrocytes obtained from defibrinated blood were the cells used. It was necessary to find some substance which would penetrate the cells slowly enough so that respiratory measurements could be made during the swelling process. Preliminary tests showed that erythritol penetrated chicken erythrocytes at 37°C. at such a rate that considerable hemolysis had occurred in about 45 minutes. Although this substance penetrated a little more rapidly than was desirable for these experiments, larger molecules could not be used because they failed to penetrate in a reasonable length of time. By lowering the temperature the rate of penetration could have been decreased, but this would also have decreased the rate of oxygen consumption. The experiments were made at a temperature of 37° ± 0.02°C., and all possible manipulations were made before the blood was added. This was done to minimize the amount of swelling which occurred before the respiratory measurements were begun.

The blood was centrifuged before using to remove as much of the serum as possible. Since small volumes of solutions had to be used, it was necessary to eliminate the osmotic effect of the serum. Although only a small amount would have been added, it would have reduced the volume of the experimental solution which could have been used. This in turn would have made the solution surrounding the cells more nearly isosmotic, which would have resulted in a smaller volume change of the cells. Oxygen consumption measurements were made using a Barcroft-Warburg microrespirometer as previously described (Hunter and Pahigian, 1940). Three vessels were used for a single experiment. The control consisted of one-half cc. of chicken erythrocytes suspended in 3 cc. of Ringer-Locke. One experimental vessel contained one-half cc. of cells in 3 cc. of 0.3 M erythritol, while the second experimental vessel contained one-half cc. of cells suspended in 3 cc. of a solution of 0.3 M erythritol in Ringer-Locke. In the first experimental solution, the cells swelled from their normal volume to their hemolytic volume. In the second experimental solution, the cells swelled from a shrunken condition (due to the loss of water into the hypertonic solution) back to their normal volume.

The results of a typical experiment are plotted in Fig. 1. The cubic millimeters of oxygen are plotted against the time in minutes. It can be seen that there is no marked change in the rate of oxygen consumption in

the experimental cells. At the beginning the shrunken cells consume oxygen at a slightly slower rate, as would be expected from previous experiments (Hunter, 1939). Although this previous work showed that the oxygen consumption was irreversibly decreased when the cells were shrunk, these cells were allowed to remain in the shrunken condition for at least 1 hour before they were swollen back to their normal volume. In the present experiments the cells began to swell back to their original volume almost

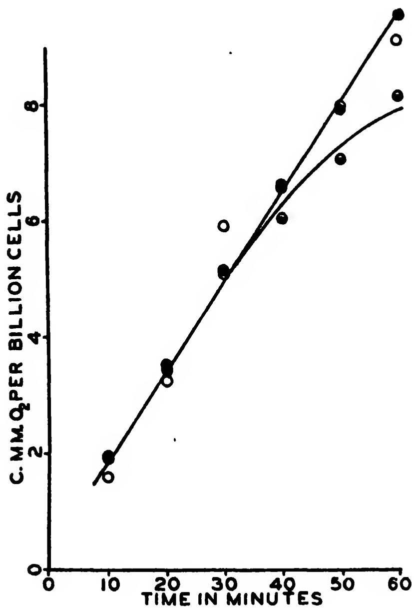

Fig. 1

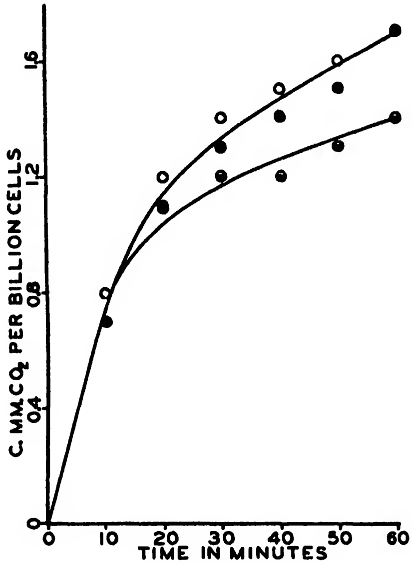

Fig. 2

Fig. 1. The effect of swelling on the oxygen consumption of chicken erythrocytes ●-Ringer-Locke; ○-erythritol in Ringer-Locke; ⊖-erythritol.

Fig. 2. The effect of swelling on the anaerobic glycolysis of beef erythrocytes. ●-Ringer-Locke; ○-erythritol in Ringer-Locke; ⊖-erythritol.

instantaneously. The fact that the oxygen consumption of the shrunken cells recovered to the normal rate when the cells began to return immediately to their normal volume is in accord with the observations of Inman (1921 *b*). This author showed that the degree of recovery of respiration was inversely proportional to the length of time the cells had remained shrunk. Except for this initial lowering of the rate, it seems to be the same as that of the control cells. This would indicate that there was no change in the aerobic respiratory activity of the cells associated with swelling from a shrunken to a normal condition. The first portion of the

curve obtained from cells suspended in erythritol solution appears to be essentially the same as the control curve. As soon as an appreciable number of cells have hemolyzed, the rate falls off as would be expected on the basis of the data presented by Michaelis and Salomon (1930). Ramsey and Warren (1930) reported a large "burst" when various types of erythrocytes were hemolyzed. In view of these experiments, it might be expected that there would be an increase in the rate of oxygen consumption as the cells hemolyzed in the erythritol solution. A further investigation by these authors (1934) demonstrated that this burst did not always appear. They also pointed out that the sudden increase in oxygen consumption depended on the plasma. Since in the present investigation only cells were used, the burst would not be expected to appear.

Since we demonstrated that aerobic oxidations are not involved when the cell membrane is stretched (*cf.* Hunter, 1936, 1937), it seemed of interest to determine if the experimental treatment would affect anaerobic glycolysis. These measurements were made in the same manner as previously described (Hunter and Pahigian, 1940). Because the oxygen in the vessels had to be displaced by a nitrogen-carbon dioxide mixture, a longer equilibration period was necessary before the readings could be taken. Consequently, some other type of cell had to be used. It was found that erythritol penetrated beef erythrocytes at a much slower rate (*cf.* Jacobs, Glassman, and Parpart, 1935). Although non-nucleated erythrocytes consume very little oxygen (Michaelis and Salomon, 1930), it has been shown that they produce lactic acid anaerobically at a considerably faster rate (Kempner, 1939). In view of these two facts, beef erythrocytes were used for the glycolysis measurements. The blood was freshly drawn from the jugular vein into a sterile bottle and defibrinated. In most experiments it was used within a few hours of drawing, while in others it was kept in an ice box for 24 hours. The results were the same in either case. In these experiments the control solution was Ringer-Locke containing 200 mg. per cent glucose and 0.03 N $NaHCO_3$. One experimental solution contained 0.3 M erythritol, 0.03 N $NaHCO_3$, and 200 mg. per cent glucose, while the second experimental solution was the same as the above, except it was m e up in Ringer-Locke. The osmotic effect of the $NaHCO_3$ and glucose was negligible since the amounts added were small, and the changes in cell volume would still occur. The Warburg vessels used in these experiments were smaller (about 6 cc.) than those used in the preceding ones. Consequently, a smaller total volume of solution was used. In some experiments 0.3 cc. of centrifuged cells was added to 1.7 cc. of solution, while in others 0.6 cc. of cells was added to 1.4 cc. of solution. Half an hour was allowed for the displacement of the oxygen by the N_2-CO_2 gas mixture.

A typical pair of curves are plotted in Fig. 2. The cubic millimeters of carbon dioxide produced are plotted against the time in minutes. It should be remembered that 1 c. mm. of carbon dioxide is equivalent to 0.004 mg. of lactic acid. It is evident that the change in cell size has no marked effect on the anaerobic breakdown of sugars until the cells begin to hemolyze.

These results are what might have been postulated on the basis of previous experiments. Without experimental evidence, however, such predictions would not have carried much weight. It should be pointed out that a mammalian erythrocyte, because of its peculiar shape, does not undergo as great a change in surface area as would at first appear. That it does undergo a certain amount of stretching, however, is not denied (Ponder and Marsland, 1935). It is reasonable to assume that because of its initial shape an avian erythrocyte when it swells would have a greater change in surface area than a mammalian erythrocyte. The fact that a change in cell size, and hence a change in the surface area, is not accompanied by any compensatory change in either oxygen consumption or anaerobic glycolysis adds further evidence testing the hypothesis of a relationship between the cell membrane and its respiratory activity.

SUMMARY

1. Oxygen consumption measurements made while a chicken erythrocyte swells show no increase over the control value.

2. There is no change in the rate of anaerobic glycolysis in beef erythrocytes when they swell.

3. The above statements are true whether the cells swell from a shrunken condition back to the normal volume, or swell from the normal to the hemolytic volume.

4. These data add a further test of the hypothesis that a relationship exists between the cell membrane and its respiratory activity.

LITERATURE CITED

Bodine, J. H., 1933, The effect of hypertonic solutions on the oxygen consumption of a developing egg (Orthoptera), *Physiol. Zool.*, **6**, 150.

Hunter, F. R., 1936, The effect of lack of oxygen on cell permeability, *J. Cell. and Comp. Physiol.*, **9**, 15.

Hunter, F. R., 1937, The effect of prolonged exposures to lack of oxygen on the permeability of the erythrocyte, *J. Cell. and Comp. Physiol.*, **10**, 241.

Hunter, F. R., 1939, The effect of hypertonic and hypotonic solutions on the respiration of chicken erythrocytes, *J. Cell. and Comp. Physiol.*, **14**, 412.

Hunter, F. R., and Pahigian, V., 1940, The effect of temperature on cell permeability and on cell respiration, *J. Cell. and Comp. Physiol.*, **15**, 387.

Inman, O. L., 1921*a*, Comparative studies on respiration. XVI. Effects of hypotonic and hypertonic solutions upon respiration, *J. Gen. Physiol.*, **3**, 533.

Inman, O. L., 1921*b*, Comparative studies on respiration. XVII. Decreased respiration and recovery, *J. Gen. Physiol.*, **3**, 663.

Jacobs, M. H., Glassman, H. W., and Parpart, A. K., 1935, Osmotic properties of the erythrocyte. VII. The temperature coefficients of certain hemolytic processes, *J. Cell. and Comp. Physiol.*, **7**, 197.

Johnson, F. H., and Harvey, E. N., 1938, Bacterial luminescence, respiration and viability in relation to osmotic pressure and specific salts of sea water, *J. Cell. and Comp. Physiol.*, **11**, 213.

Kempner, W., 1939, The nature of leukemic blood cells as determined by their metabolism, *J. Clin. Inv.*, **18**, 291.

Michaelis, L., and Salomon, K., 1930, Respiration of mammalian erythrocytes, *J. Gen. Physiol.*, **13**, 683.

Ponder, E., Personal communication.

Ponder, E., and Marsland, D., 1935, The escape of hemoglobin from the red cell during hemolysis, *J. Gen. Physiol.*, **19**, 35.

Ramsey, R., and Warren, C. O., 1930, The rate of respiration in erythrocytes, *Quart. J. Exp. Physiol.*, **20**, 213.

Ramsey, R., and Warren, C. O., 1934, The oxygen consumption of rabbit red cells during lysis, with some observations on the oxygen consumption of plasma, *Quart. J. Exp. Physiol.*, **24**, 153.

Ray, G. B., 1927, The effect of hyper- and hypotonic solutions on oxidations by the red blood cells, *Proc. Soc. Exp. Biol. and Med.*, **25**, 30.

Tipton, S. R., 1933, Factors affecting the respiration of vertebrate red blood cells, *J. Cell. and Comp. Physiol.* **3**, 313.

STUDIES ON PITUITARY LACTOGENIC HORMONE

III. Solubilities of Sheep and Beef Hormones*

By CHOH HAO LI, WILLIAM R. LYONS, and HERBERT M. EVANS

(From the Institute of Experimental Biology and the Department of Anatomy, University of California, Berkeley)

(Received for publication, August 13, 1940)

INTRODUCTION

Northrop and coworkers (1–3) believe that solubility studies constitute the most sensitive and reliable test for determining the homogeneity of proteins and have used them successfully as a test for purity of their crystalline enzyme preparations. Herriott, Desreux, and Northrop (4) were able to show that a number of pepsin solutions that appeared to be homogeneous in electrophoresis experiments contained several protein components as indicated by solubility studies. Steinhardt (5) has recently shown that in the case of some proteins difficulties may attend the application of the test.

Cohn and coworkers (6, 7) have carried out protein solubility studies using the salting-out effect of electrolytes in buffer solutions. If proteins are pure, a plot of the logarithm of solubility against ionic strength may appear to be a straight line. Slopes and intersects to the ordinate are used in characterizing the protein.

The solubility method is not only useful in determining the purity of a preparation but is a sensitive method for distinguishing species specificity of proteins (8). Our preparations of pituitary lactogenic hormone have been demonstrated recently to act as a homogeneous substance in the Tiselius apparatus (9), and preparations of beef and sheep hormone were indistinguishable (10) electrophoretically. Bischoff and Lyons (11) were not able to differentiate the hormone prepared from beef or sheep pituitary by certain immunological methods. It seemed worth while, therefore, to investigate the solubility behavior of our beef and sheep lactogenic hormones in order to determine not only whether they were pure but

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also whether they were species specific. The beef and sheep lactogenic hormones used in this work were prepared in essentially the same manner as previously described (12).

A. Solubility in NaCl-HCl Solution

Preparation and Solvent.—L299S[1] contained approximately 30 International Units per mg. The isoelectric precipitate (about 300 mg.) was washed twice with 40–50 cc. of solvent. The washed precipitate was used for the solubility experiments. The solvent was made with 0.302 M NaCl in 0.01 N HCl solution and contained 2 per cent butanol. The pH of the solution was 2.02[2] (glass electrode).

Methods.—The precipitate which had been washed twice with the solvent was broken into a fine suspension by agitation[3] for 15 hours in a closed test tube (12 × 100 mm. Pyrex).

Varying amounts of this suspension were distributed into test tubes and each tube was then filled with the solvent. The technique to displace the remaining air bubbles was essentially the same as that described by Kunitz and Northrop (13). The tubes were then agitated on the wheel for 2 or 3 days. The solutions were filtered through a Whatman filter paper (No. 42) and the filtrate analyzed for nitrogen. Nitrogen was determined by a micro-Kjeldahl method and the results represent averages of two or more determinations.

The amount of protein dissolved was sometimes checked by Folin's phenol reagent (14) and carried out in the following manner. To 1 cc. of the filtrate, diluted to 10 cc., were added 2 cc. of 1 M NaOH and 3 cc. 1:3 Folin's reagent. The mixture was kept in an oven at 40°C. for 15 minutes. The color produced was measured in the Cenco photelometer using the red filter. The protein content was then read from a standard curve which had been constructed by using known quantities of protein in the same manner. The amount of nitrogen was obtained by assuming that the hormone contained 14.52[4] per cent nitrogen.

Results.—Results are given in Fig. 1. The curve shows that the preparation obeys strictly the requirements of the phase rule for a single substance. It may be noted that the initial slope of the curve is 1, indicating that the solutions were perfectly clear before the appearance of the solid phase. This

[1] Throughout this paper *S* indicates sheep and *B*, beef origin of the pituitary lactogenic preparations.

[2] In this connection, the stability of lactogenic hormone in acid solutions was studied. 10 mg. L299S were dissolved in 5 cc. 0.1 M HCl. The mixtures were kept at room temperature (20–21°C.) for 1, 2, and 3 days in the presence of 2 per cent butanol as preservative. Before being assayed solutions were neutralized and it was found that there was no difference in potency in the 1 and 2 day samples, whereas the 3 day sample showed a loss in potency of about 50 per cent.

[3] The test tubes were clamped on a revolving wheel; two glass beads were placed in each tube to agitate the material.

[4] This value is low because corrections were not made for ash and moisture.

fact as well as our determination that the biological potency of the soluble protein was the same as that of the insoluble protein in saturated solutions presents further evidence for homogeneity of the hormone.

B. Solubility in Water

Water is well known to be the most favorable solvent for solubility studies because interactions which sometimes take place between salts and their

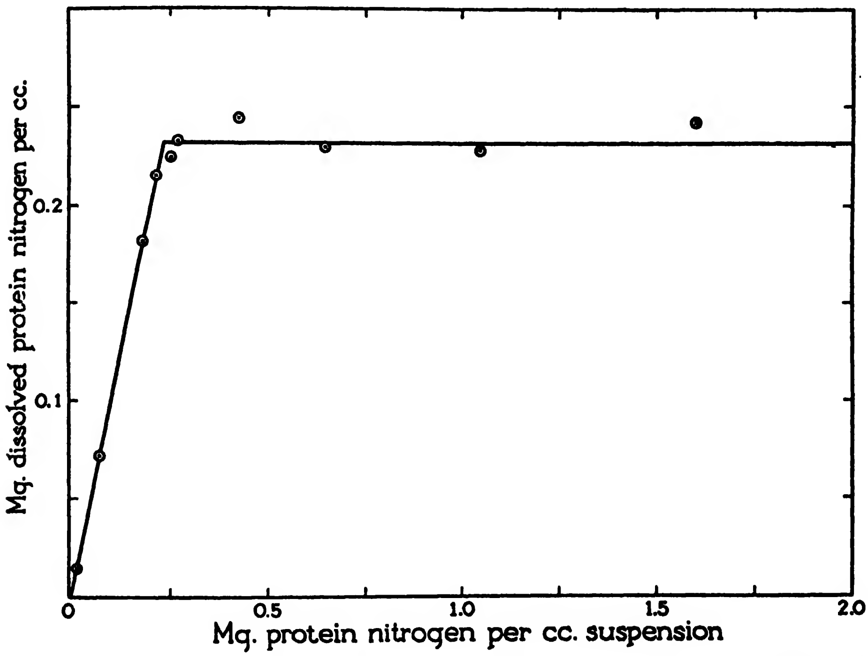

FIG. 1. Solubility of sheep lactogenic hormone preparation (L299S) in 0.302 M NaCl solution of pH 2.02 at 21–22°C.

saturating body are eliminated. As pointed out by Cohn (16), the solubility in water of a pure protein may be considered as a fundamental, physicochemical constant which may be used in identifying and in classifying proteins. However, owing to the difficulty in removing the last trace of salt, acid, and alkali from isoelectric proteins there are very few pure proteins which have been investigated as to their homogeneity, by the method of solubility in water.

L294B was an isoelectric preparation obtained from beef pituitaries. Approximately 1 gm. of wet isoelectric precipitate was triturated three times with 50 cc. water. Two solubility experiments were carried out using different quantities of the washed precipi-

than the same volume of water. The test tubes (as in experiments of section A) were agitated on the wheel for 2 days at room temperature; the suspensions were then rocked in a cold room at a temperature of 7–8°C. for another 2 days. The solubility of isoelectric beef lactogenic hormone in water (at 7–8°C.) was found to be 0.102 gm. per liter in the tube containing the smallest amount of solid, while in the tube containing seven times that amount of saturating body, the solubility was practically the same (0.104 gm. per liter). This indicates that the preparation behaved as a single substance. The low solubility of the protein suggests that it has a low dissociation constant and possibly that it has a small number of free polar groupings in the molecule.

C. Salting Out Effect of NaCl in Acid Solution

The influence of salts on the solubility of proteins has been used not only for separation and purification purposes but for the characterization of proteins. Cohn (17) has shown that solubility is defined by an equation of the form

$$\log S = \beta - K_s\mu$$

in which K_s is a salting-out constant characteristic of the salt employed, β an intercept constant characteristic of the saturating substance, μ the ionic strength per 1000 gm. of water, and S the solubility of the protein in gm. per liter. Using this technique, Green, Cohn, and Blanchard (18) were able to show the species specificity of horse and human carboxyhemoglobins. Since species specificity has not hitherto been shown to exist in the case of lactogenic hormone, it seemed interesting to attempt with the aid of this method a differentiation between our beef and sheep lactogenic preparations.

Experimental.—L287B and L288S were acetone-dried isoelectric precipitates. They both contained approximately 30 I.U. per mg. 20 mg. of L287B or L288S were dissolved in 2 cc. of 0.01 N HCl and to this were added 3 cc. of 0.01 N HCl containing different amounts of NaCl.[5] The test tube was then shaken slowly for about 2 hours at room temperature. The suspension was filtered and the filtrate was analyzed for nitrogen as described in section A.

Results.—The behavior of these two lactogenic hormones is graphically represented in Fig. 2. These results show that the solubility of both L287B and L288S followed the rule represented by the equation previously given. Furthermore, sheep lactogenic hormone was found to be more soluble than the beef hormone in these acid solutions.[6] It is of interest to note that the

[5] Since solubility is a function not only of the concentration of the salt, but of the pH and the temperature, it is very important to make up the solvent with extreme accuracy. This has not been attempted in the present experiments since the results were intended primarily for the purpose of comparing sheep and beef hormones rather than establishing characteristic absolute values for the solubility.

[6] In experiments using citrate buffer (0.1 M, pH = 6.36) beef lactogenic hormone has been found to be more soluble than sheep.

slopes (K_s) of the two curves are almost the same whereas the intercept constants (β) exhibit differences.

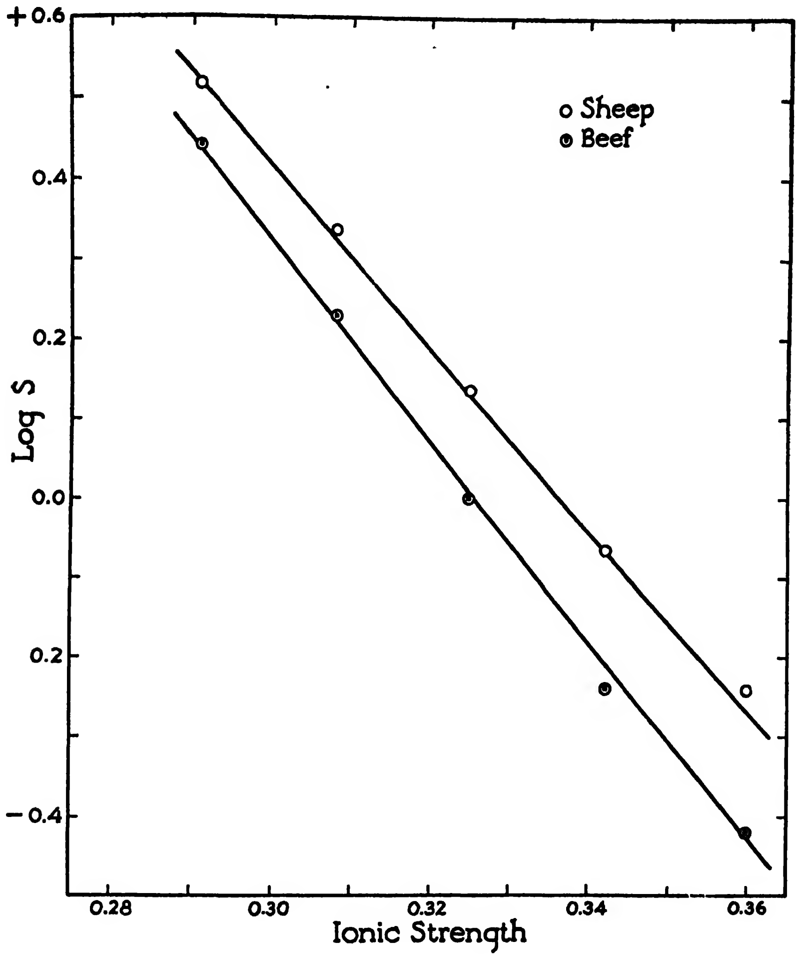

FIG. 2. Solubilities of beef and sheep lactogenic hormone (L287B) and (L288S) in different concentrations of NaCl of 0.01 N HCl solutions at 21–22°C.

It may also be seen that NaCl proved to be a very effective precipitant for the hormone dissolved in dilute HCl.[7] We have always observed that the lactogenic hormone is very soluble in this solvent, but its solubility is greatly decreased in the presence of salts. The solubility in alkaline solu-

[7] The addition of HCl to a strength of 0.5 M causes the precipitation of lactogenic hormone.

tion is, however, not so much affected by the presence of NaCl. Thus, the precipitation of lactogenic hormone began only when 32 gm. of NaCl were added to 100 cc. 0.01 N NaOH solution; on the other hand, the hormone began to precipitate out in 0.01 N acid solution when 1.5 gm. of NaCl were added. Solubility is a measure of the interaction between protein dipoles and the ions of a salt and the difference in behavior of the protein in acid and basic solutions may be attributed, in part, to the fact that the dissociation constants of certain groups are much higher above than below the isoelectric point.

D. DISCUSSION

It is generally agreed that crystalline proteins are not always homogeneous with respect to sedimentation, electric charge, and solubility, but ultracentrifugation, electrophoresis, and solubility studies also have limitations. Since proteins fall into molecular weight classes (19), and so many proteins have molecular weights ranging from 34,000 to 42,000, the ultracentrifuge has often proved unsatisfactory in characterizing the purity of a protein. Furthermore, a single boundary in the Tiselius apparatus does not necessarily signify a pure substance. Solubility studies constitute the best available method for determining homogeneity of a protein, especially if they are carried out with a variety of solvents, and due regard is given to the possibility that solubility anomalies may occur under certain circumstances (5, 20, 21).

Although we have not yet been able to secure the pituitary lactogenic hormone in *uniform* crystalline state, our preparations behave in electrophoresis experiments (9) as a single substance. The solubility studies herein reported also failed to disclose more than a single component. Preliminary work on ultracentrifugation of the lactogenic hormone has so far shown it to be homogeneous, but further data have yet to be collected.

The species specificity of pituitary lactogenic hormone as demonstrated in this paper is particularly interesting. In our previous work we (10) were not able to distinguish the sheep pituitary hormone from that of beef pituitary in electrophoresis experiments. Bischoff and Lyons (11) were also unable to differentiate them through the use of precipitin, anaphylaxis, or the Dale and Arthus reactions. An analogous situation has been found to be the case with pepsin (22).

SUMMARY

The solubility of sheep pituitary lactogenic hormone in 0.302 M NaCl at pH 2.02 (solution in HCl) has been determined at room temperature.

It showed a constant solubility in the presence of a considerable excess of the solid phase, an indication that the preparation contained but one component.

Beef lactogenic hormone showed a constant solubility in distilled H_2O at 7–8°C in the presence of excess of the solid phase.

The salting-out effect of NaCl in acid solution of both beef and sheep hormones has been studied at room temperature. In these studies both preparations behaved as pure substances, but they exhibited differences in solubility, thus indicating a species specificity.

BIBLIOGRAPHY

1. Northrop, J. H., *J. Gen. Physiol.*, 1930, **13**, 739.
2. Northrop, J. H., and Kunitz, M., *J. Gen. Physiol.*, 1932, **16**, 267.
3. Northrop, J. H., Crystalline enzymes, Columbia Biological Series, No. 12, New York, Columbia University Press, 1939, p. 28.
4. Herriott, R. M., Desreux, V., and Northrop, J. H., *J. Gen. Physiol.*, 1940, **23**, 439.
5. Steinhardt, J., Cold Spring Harbor symposia on quantitative biology, Cold Spring Harbor, Long Island Biological Association, 1938, **6**, 301.
6. Cohn, E. J., *Chem. Rev.*, 1936, **19**, 241.
7. Ferry, R. M., Cohn, E. J., and Newman, E. S., *J. Am. Chem. Soc.*, 1938, **60**, 1480.
8. Landsteiner, K., and Heidelberger, M., *J. Gen. Physiol.*, 1923, **6**, 131.
9. Li, C. H., Lyons, W. R., and Evans, H. M., *J. Gen. Physiol.*, 1940, **23**, 433.
10. Li, C. H., Lyons, W. R., and Evans, H. M., *J. Am. Chem. Soc.*, 1940, **62**, 2925.
11. Bischoff, H. W., and Lyons, W. R., *Endocrinology*, 1939, **25**, 17.
12. Lyons, W. R., Cold Spring Harbor symposia on quantitative biology, Cold Spring Harbor, Long Island Biological Association, 1937, **5**, 198.
13. Kunitz, M., and Northrop, J. H., *Compt.-rend. trav. Lab. Carlsberg*, 1937, **22**, 293.
14. Folin, O., and Ciocalteu, V., *J. Biol. Chem.*, 1927, **73**, 627.
15. Cohn, E. J., Berggren, R. E. L., and Hendry, J. L., *J. Gen. Physiol.*, 1924–25, **7**, 81.
16. Cohn, E. J., *Physiol. Rev.*, 1925, **5**, 349.
17. Cohn, E. J., *Ann. Rev. Biochem.*, 1935, **4**, 136.
18. Green, A. A., Cohn, E. J., and Blanchard, M. H., *J. Biol. Chem.*, 1935, **109**, 631.
19. Svedberg, T., *Ind. and Eng. Chem., Analytical Edition*, 1938, **10**, 113.
20. Linderstrøm-Lang, K., *Ann. Rev. Biochem.*, 1939, **8**, 38.
21. Northrop, J. H., and Kunitz, M., *J. Gen. Physiol.*, 1930, **13**, 781.
22. Northrop, J. H., Crystalline enzymes, Columbia Biological Series, No. 12, New York, Columbia University Press, 1939, p. 31.

EFFECTS OF HEXYLRESORCINOL ON VALONIA

By W. J. V. OSTERHOUT

(From the Laboratories of The Rockefeller Institute for Medical Research)

(Received for publication, September 12, 1940)

Experiments on *Nitella* have shown considerable resemblance[1] between the effects of hexylresorcinol and those of guaiacol. This resemblance is even more striking in *Valonia*.

Fig. 1 shows[2] the effect of 0.0006 M hexylresorcinol in sea water. At the start the cell had a negative[3] P.D. of 6 mv. After a latent period of about 15 seconds the curve fell, indicating a change of P.D. in a positive direction amounting to 34 mv. After this the curve rose gradually almost to the original level. The course of the curve is like that seen under the influence of guaiacol[4] and may be due to similar causes.

The curve in Fig. 1 represents fairly well the average behavior but great variation is possible. With curves showing a sharp drop,[5] as in Fig. 1, the latent period may vary from 3 to 40 seconds and the P.D. may become positive to the extent of from 17 to 50 mv. (the average value was 28 mv.). Recovery requires from 35 to 100 seconds.

If the rise of the curve, showing a loss of positive P.D., were due to injury we should expect the final P.D. to be zero. This was not the case. The curve as a rule flattened out at a positive value of the P.D. and showed no sign of going to zero. As a rule it failed to rise to the starting point. In most cases the cells appeared normal on subsequent days and behaved nor-

[1] Osterhout, W. J. V., *J. Gen. Physiol.*, 1939–40, **23**, 569.

[2] The experiments were made on *Valonia macrophysa*, Kütz., using the technique described in former papers (Osterhout, W. J. V., *J. Gen. Physiol.*, 1936–37, **20**, 13; regarding the amplifier see Hill, S. E., and Osterhout, W. J. V., *J. Gen. Physiol.*, 1937–38, **21**, 541).

The temperature varied from 20 to 25°C.

There was no sign of injury unless otherwise stated.

Grateful acknowledgment is made to the firm of Sharp and Dohme of Glenolden, Pa. for their generous gift of hexylresorcinol.

[3] The P.D. is negative when the positive current tends to flow from the sea water across the protoplasm to the sap. The P.D. is usually negative by 5 to 10 mv.

[4] Osterhout, W. J. V., *J. Gen. Physiol.*, 1936–37, **20**, 13.

[5] With some cells the curve fell very gradually and the P.D. did not become more than 12 mv. positive. In such cases there was little or no recovery.

mally when subjected to experimental treatment. One does not therefore get the impression that the rise of the curve was due to injury in the usual sense of the word.

For convenience we may speak of the rise of the curve as "recovery" with the understanding that complete "recovery" does not mean that the cell returns to its original state. That it does not is shown by tests which will now be described.

a. The Potassium Effect.—In normal cells transferred from sea water to "0.3 M K sea water" the P.D. became more negative by 10 to 31 mv.[6] But

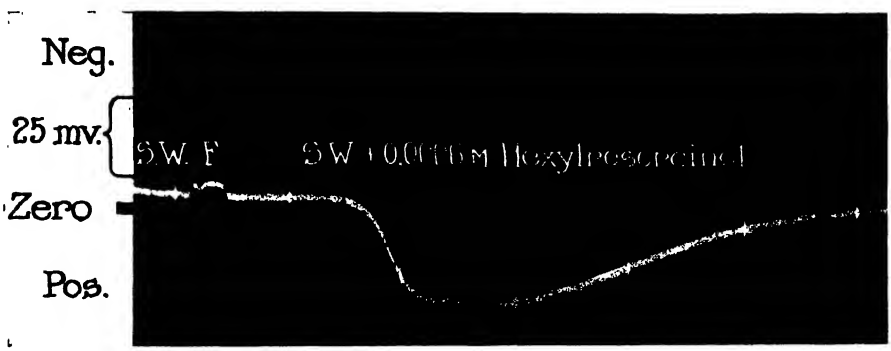

FIG. 1. At the start the cell in sea water showed a negative P.D. of 6 mv. When the cell was removed from sea water the curve jumped to *F*, recording the free grid of the amplifier. When transferred to sea water + 0.0006 M hexylresorcinol there was a latent period of about 15 seconds after which the curve fell (the P.D. becoming positive) and then rose: the rise is called "recovery" for convenience but this does not mean that the cell is returning to its original condition.

Time marks 15 seconds apart.

Temperature 25°C.

in the presence of 0.0006 M to 0.003 M hexylresorcinol this potassium effect largely disappeared. A similar result was obtained with guaiacol.[4]

The 0.3 M K sea water was made by mixing equal parts of sea water and 0.6 M KCl (the consequent dilution of Na^+, Mg^{++}, and Ca^{++} is considered of no importance, especially in such short experiments).

The cells were exposed to 0.3 M K sea water for 5 minutes or less and then returned to sea water. After standing from 3 to 24 hours in sea water they were transferred to sea water + hexylresorcinol and left for several minutes. They were then placed in 0.3 M K sea water + hexylresorcinol in order to measure the potassium effect.

b. The Dilution Effect.—When normal cells were transferred from sea water to sea water plus an equal volume of 1.1 M glycerol (containing 0.02

[6] *Cf.* Damon, E. B., *J. Gen. Physiol.*, 1937–38, **21**, 383.

M $CaCl_2$ + 0.012 M KCl) the change of P.D. was about 5 mv. in a negative direction.[6] We may regard this as indicating that the mobility of Na^+ (u_{Na}) is less than that of Cl (v_{Cl}).

When a similar experiment is made with sea water containing 0.0006 M to 0.003 M hexylresorcinol there is no change of P.D. or a small change in a positive direction. We may regard this as indicating that the order $v_{Cl} > u_{Na}$ has been changed to $u_{Na} = v_{Cl}$ or to $u_{Na} > v_{Cl}$. A similar result is obtained with guaiacol.[4]

In view of the chemical resemblance of guaiacol ($C_6H_4(OH)OCH_3$) to hexylresorcinol ($C_6H_3(OH)_2\ C_6H_{13}$) it is not surprising that their effects are similar. But hexylresorcinol is as effective at 0.0006 M to 0.003 M as guaiacol at 0.01 M to 0.03 M. It may be noted that the former is much more surface-active.

It is most interesting to find that both substances can influence the behavior of inorganic ions so markedly. This is presumably due to their effects on mobilities and on partition coefficients. This opens up an interesting field of research which can be profitably pursued by the use of models. A beginning has already been made in this direction.[7]

SUMMARY

The effects on *Valonia* of guaiacol and hexylresorcinol are similar but the latter is more effective.

Both substances lower or abolish the potassium effect; *i.e.*, the ability of the cell to distinguish electrically between Na^+ and K^+.

Both substances change the order of mobilities so that $v_{Cl} > u_{Na}$ becomes $u_{Na} > v_{Cl}$ or $u_{Na} = v_{Cl}$.

[7] Osterhout, W. J. V., Some models of protoplasmic surfaces, in Cold Spring Harbor symposia on quantitative biology, Cold Spring Harbor, Long Island Biological Association, 1940, **8**, 51.

AN X-RAY AND CRYSTALLOGRAPHIC STUDY OF RIBONUCLEASE

BY I. FANKUCHEN*

(From the Department of Physics, Massachusetts Institute of Technology, Cambridge)

(Received for publication, September 24, 1940)

A new crystalline protein of low molecular weight, ribonuclease, has been isolated and described by Kunitz (1, 2). A preliminary study of this protein has now been made with x-rays and with the polarizing microscope.

The material studied was crystallized from an ethanol-water solution. The crystals were dried in air without appreciable deterioration and the measurements here described were made with these air-dried crystals. They are long, thin needles, orthorhombic, elongated along the "*c*" axis. The prism faces are (110) and ($1\bar{1}0$) and include an angle of 70°. The other two axes bisect the angles of the cross section, "*a*" bisecting the obtuse angle and "*b*" the acute. The extinction as observed in the polarizing microscope is, of course, straight; α is along a, β along b, and γ along c. The crystal is positive and the optic axial angle is about 65° measured in air and 74° in glycerine.

X-ray oscillation films were taken about all three crystallographic axes. These give the following values for the unit cell; $a = 36.6$ A, $b = 40.5$A, and $c = 52.3$ A. The space group appears to be $P2_12_12_1$, 4 molecules per unit cell, each molecule without symmetry in a general position. The density of the air-dried crystals was measured to be 1.341 ± 0.002, by floating them in a mixture of methylene chloride and carbon tetrachloride. The molecular weight has been computed from these data assuming 4 molecules per unit cell. The cell volume is 77,300 A^3 and this gives a molecular weight of 15,700 ± 300. This value is an upper limit as no correction has been made for solvent of crystallization.[1]

* National Research Fellow in Protein Chemistry.

[1] Dr. A. Rothen (3) has found the specific volume of ribonuclease to be 0.709. The density of the air-dried crystals corresponds to a value of 0.746. He suggests that this difference may be due mainly to hydration and a computation based on this difference gives a value for the hydration of 12.7 per cent. An estimate of the molecular weight of the anhydrous protein can then be obtained by reducing the x-ray value of 15,700 by 12.7 per cent. This gives 13,700 which may be compared with the molecular weights reported from sedimentation and diffusion, 13,000, and from osmotic pressure measure-

These air-dried crystals are unusually perfect and give sharp diffraction spots down to 2 A, whereas no other dried protein crystal has thus far given reflections below 5 A (chymotrypsin). This, in conjunction with the low molecular weight, suggests that a study of x-ray intensities, similar to that of Crowfoot on insulin (4, 5), may give useful information about the internal structure of the ribonuclease molecule. A preliminary investigation of this character is now in progress.

REFERENCES

1. Kunitz, M., *Science*, 1939, **90**, 112.
2. Kunitz, M., *J. Gen. Physiol.*, 1940, **24**, 15.
3. Rothen, A., *J. Gen. Physiol.*, 1940, **24**, 203.
4. Crowfoot, D., *Proc. Roy. Soc. London, Series A*, 1938, **164**, 580.
5. Crowfoot, D., and Riley, D. P., *Nature*, 1939, **144**, 1011.

ments, 15,000. Computations of this kind depend on comparatively small differences of specific volume and can be accepted only with reserve. They are certainly less satisfactory than direct determinations of the hydration of the crystals, but lacking such determinations do serve to give an estimate of the hydration.

THE FLICKER RESPONSE CONTOUR FOR PHRYNOSOMA (HORNED LIZARD; CONE RETINA)

BY W. J. CROZIER AND ERNST WOLF

(*From the Biological Laboratories, Harvard University, Cambridge*)

(Received for publication, August 26, 1940)

I

The lizard *Phrynosoma* was found by Loeb (1907, 1918) to exhibit in a particularly clear way the effects of moving visual images upon forced compensatory reactions; head nystagmus of visual origin is easily demonstrated when a white paper cylinder with black vertical stripes is rotated about the animal, under ordinary daylight illumination. "Optokinetic" reactions of this type are readily obtained with other saurians also (Loeb, 1891; Trendelenburg and Kühn, 1908; Schlieper, 1927; Ohm, 1931; Ehrenhardt, 1937; Crozier and Wolf, 1938–39). A particular interest of these reactions of diurnal lizards such as *Phrynosoma* is due to the fact that the retina (Detwiler and Laurens, 1920; Keeler, 1930) contains sensory elements held to be of only one general histological type, namely single cones (Garten, 1907; Rochon-Duvigneaud, 1917; Menner, 1929; Walls, 1934; Verrier, 1935), which seems to be true, however, of the nocturnal form *Heloderma* as well (Walls, 1934). With this sensorially simplex constitution of the retina of *Phrynosoma* the data on its electrical responses to photic stimulation are not inconsistent (Meservey and Chaffee, 1927; Chaffee and Sutcliffe, 1930). Although no rods are present, the pronounced fovea contains attenuated cones which lack the oil droplet characteristically occurring in the extra-foveal cones (Detwiler and Laurens, 1920). A large, conical, deeply pigmented pecten is present in the *Phrynosoma* eye; it has been suggested recently (Menner, 1938) that this structure in the bird's eye may play a part in increasing the excitatory effect of moving images, but we find in the responses to flicker no evidence as to this, even at low flash frequencies (equal light and dark times), in the case of *Phrynosoma* as well as with birds we have recently studied; when the t_L/t_D ratio is changed, such evidence is obtained (Crozier and Wolf, 1940–41*b*).

The expectation for animals with retinal receptor elements of single type is that the flicker response contour should be a simplex symmetrical probability integral (F – log I; Crozier, Wolf, and Zerrahn-Wolf, 1938 *a*,

1938–39 *b*), although the activity of the iris may introduce a complication in some cases (*cf.* Crozier and Wolf, 1938–39 *b*). Employing the striped cylinder technic and the procedures previously described (Wolf and Zerrahn-Wolf, 1935–36; Crozier, 1935–36; Crozier, Wolf, and Zerrahn-Wolf, 1936–37 *a*; Crozier and Wolf, 1939–40 *b*), it has been found that several vertebrates with simplex (rod or cone) constitution of the retina provide simplex performance contours relating flash frequency to flash illumination critical for forced head nystagmus; whereas the corresponding contours for vertebrates having both rods and cones are characteristically duplex (Crozier, Wolf, and Zerrahn-Wolf, 1938 *a*; Crozier and Wolf, 1938 *a*, 1939). It is desirable to extend the factual basis for certain considerations arising in connection with these facts. A significant general theory of visual duplexity in vertebrates requires, among other things, that something more be known about homologous performance contours of animals having only rods and of types having only cones. The behavior of *Phrynosoma* provides data significant for this end. The important points have to do with (1) the analytical shape of the performance contour, and (2) the magnitudes of its parameters in comparison with those of other "cone" visual performance curves.

II

Phrynosoma cornutum was used in these experiments. A considerable stock was obtained from a commercial supply company. The individuals used were 10 to 15 cm. in total length. They were kept in a large cage with western exposure, and fed on captured flesh-flies and on *Drosophila* from cultures. During daylight hours they were quite active. For the observations each selected individual was put in a thin-walled crystallizing dish covered by a watch glass; the cover was so fastened as to be firmly clamped on, but with space around the edge to provide for circulation of air. When surrounded by the rotating striped cylinder with sufficiently intense trans-illumination, a slow movement of the head ("railroad" nystagmus) in the direction of rotation is periodically interrupted by swift returns of the head to the initial position in line with the axis of the body. In this reaction the front legs are extended and the body is elevated and gradually swung in the direction of the motion of the stripes. In some cases locomotion in the stripe-movement direction is observed.

The initiation of head nystagmus as the flash intensity is increased with F constant provides the index of threshold response to ("recognition" of) the light-dark cycle. In these observations the light and the opaque spaces on the cylinder were equal (*i.e.*, $t_L/t_D = 1$). For quantitative observations preliminary dark adaptation is essential; but in darkness *Phrynosoma* quickly becomes inactive, the eyes are closed, and the general condition approximates that of tonic immobility. After the period of dark adaptation it was consequently desirable to tilt the container in order to cause the animal to "awaken" and open the eyes as a consequence of the righting reflexes. Preliminary tapping of the glass container also helps. Very often these animals close the eyes

immediately after having responded once as result of increasing the light intensity to the critical level, and then pass into a kind of general rigidity. This is in marked contrast to the persistence of head nystagmus within a rotated striped cylinder in an ordinarily illuminated room. In the dark room, within the trans-illuminated cylinder, it was usually difficult to secure even three successive critical responses without arousing the lizard from immobility. Prolongation of the period of dark adaptation increased these difficulties. Under such conditions the determinations of critical flash intensity are bound to be more erratic, and the values of P.E.$_{1I_1}$ more irregular, than with the other animals we have thus far used. In general (*cf.* Table I) the variation index for I_1 is of the order of 5 per cent of I_m, but it is irregular; to the reasons for this already mentioned it is to be added that not the same ten individuals were used throughout the experiment. The measurements are therefore comparatively deficient in the matter of homogeneity.

In other sets of observations injections of adrenalin, strychnine, and picrotoxin were administered to groups of individuals, chiefly to test possible improvement of responsiveness. No conditions were found, however, which served to prevent the effect of continued darkness or dim illumination in producing the gradual stupor and immobilization.

III

The observations secured with *Phrynosoma* are summarized in Table I. They are given as log mean critical intensities (millilamberts), with the P.E.'s of the dispersals of the individual ten means of three observations on each of ten individuals. The temperature was 27.5° ± 0.3°. As shown in Fig. 1, the data of Table I are well described by a normal probability integral in log I_m. The maximum to which the probability grid in Fig. 1 is computed is $F_{max.}$ = 42.4 flashes per second. It is notable that there is no "iris effect" such as previously found with the gecko (Crozier and Wolf, 1938–39 *b*).

Among the response contours with which that for *Phrynosoma* may be compared the closest parallel is that given by the "cone" segment for fishes of the *Platypoecilius* group (data in Crozier, Wolf, and Zerrahn-Wolf, 1937, 1937–38 *a*, 1938 *b*; Crozier and Wolf, 1938 *a*, *b*, 1938–39 *a*, 1939, 1939–40 *a*). Fig. 2 exhibits the general similarity of these curves. With the measured contours for other vertebrates greater differences are apparent. The cone curve for *Rana* (Crozier and Wolf, 1939–40 *b*), and the simplex curve for the turtle *Pseudemys* (cone retina; Crozier, Wolf, and Zerrahn-Wolf, 1938–39 *a*) are also traced in Fig. 2, as well as the simplex curve for the gecko *Sphaerodactylus* (rod retina; Crozier and Wolf, 1938 *b*). It is apparent that greater differences are found in the shape constants and inflection levels of the cone curves for different animals than between the "rod" and cone curves of turtle and gecko. In the fishes we have studied the cone segments range in form (steepness) between that for *Platypoecilius* (Fig. 2) and that for *Fundulus* (Crozier and Wolf, 1939–40 *e*) which has a

slope very close to that for the turtle *Pseudemys*. On the other hand the forms of the cone curves for the frog and for the newt *Triturus* (Crozier and Wolf, 1939–40 *d*) are very closely alike.

In making these comparisons we are disregarding differences of temperature involved in the several cases. This is chiefly because our major interest is with the form and the maximum F for the different curves. We have abundant evidence that the shape constants ($\sigma'_{\log I}$) and the values of $F_{max.}$ are not dependent on the temperature (Crozier, Wolf, and Zerrahn-Wolf, 1936–37 *b*, 1938–39 *a*; Crozier and Wolf, 1939, 1939–40 *a*, *c*). The

TABLE I

Mean critical intensities, as log I_m (millilamberts), for response to visual flicker by the horned lizard *Phrynosoma* at 27.5° ± 0.3°, with $t_L = t_D$; 30 observations (three on each of ten individuals) at each flash frequency $F/sec.$; P.E.$_1$ is the P.E. of the dispersions of the individual means.

F	log I_m	log P.E.$_{1I_1}$
2	$\bar{1}.2499$	$\bar{2}.3320$
5	$\bar{1}.5739$	$\bar{2}.0917$
10	$\bar{1}.8116$	$\bar{1}.6532$
	$\bar{1}.9098$	$\bar{2}.3687$
15	0.2243	$\bar{2}.7950$
20	0.4283	$\bar{2}.9070$
	0.3454	$\bar{1}.9848$
25	0.5459	$\bar{1}.1038$
30	0.8558	$\bar{1}.7700$
	0.7302	$\bar{1}.3911$
35	1.1136	$\bar{1}.7999$
38	1.3730	$\bar{1}.9785$
42	2.0330	0.1204

data basic to Figs. 2 and 3 were obtained at two main temperatures: for gecko and for *Phrynosoma* $t° = 27.5$; for the others, excepting man (37.5°), the temperature is 21.5°. We do not know the specific temperature characteristics for the shift of the abscissa of inflection, except for *Pseudemys* (Crozier, Wolf, and Zerrahn-Wolf, 1938–39 *a*, Crozier and Wolf, 1939–40 *c*) and *Platypoecilius* (Crozier and Wolf, 1939–40 *a*). We do, however, know that in all seven cases examined elevation of temperature moves the $F - \log I$ curve toward lower intensities, without changing its shape. Consequently the unusually high value of the abscissa of inflection in the *Phrynosoma* ($\tau' = 0.42$; *cf.* Fig. 3) is not due to the high temperature prevailing during the observations. Chaffee and Sutcliffe (1930) noted that for the electrical responses of the *Phrynosoma* retina the intensity thresholds

were much higher than for the frog eye. The (cone) curve for certain birds (42.3°) we are now engaged in studying is not so steep as that for *Phrynosoma* and its τ' is very much lower ($\tau' = ca.\ \bar{3}.70$).

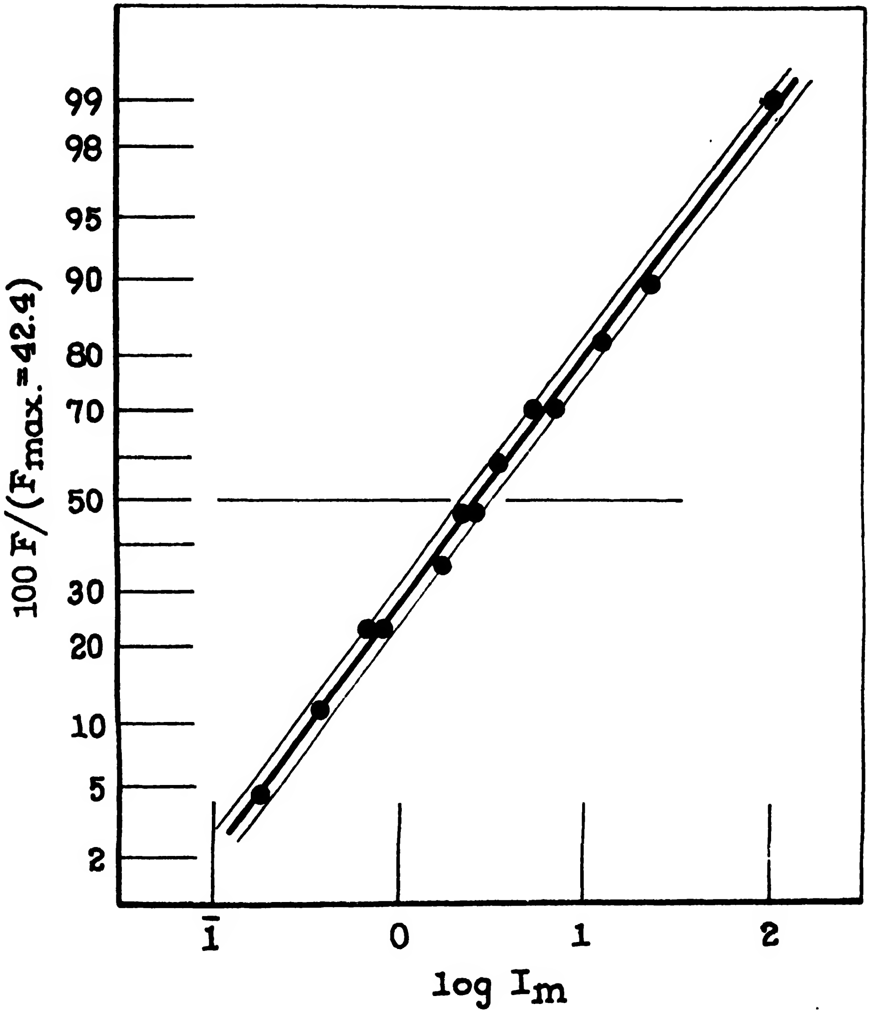

FIG. 1. Flash frequency F and log mean critical flash intensity for reaction of *Phrynosoma* to flicker; light time = $0.5/F$; temperature = 27 5°; F given as percentage of $F_{max.}$ = 42.4/sec. on the ordinate of a normal probability grid.

The maximum value of F to which response is obtainable is a function of retinal area (*cf.* Crozier, Wolf, and Zerrahn-Wolf, 1937–38 *c*), in a given animal. To what extent this is true in comparing different animals is of

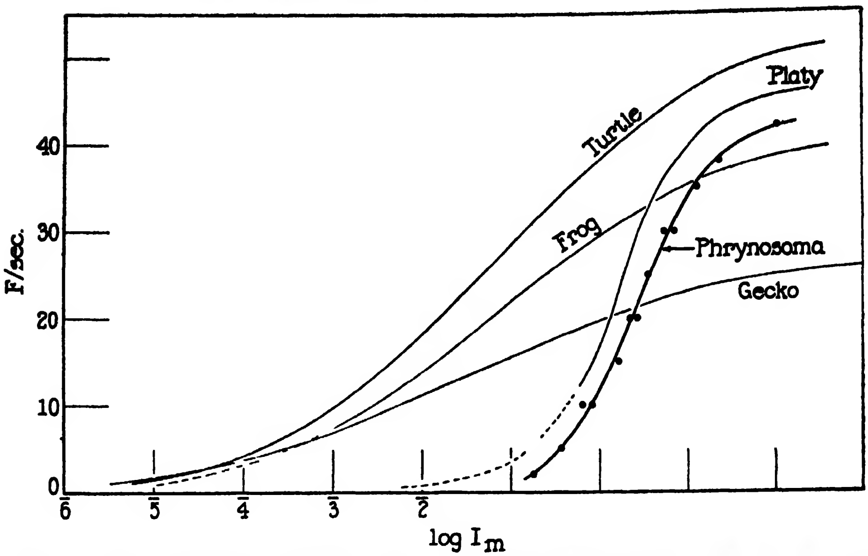

FIG. 2. The probability integral of Fig. 1 for the observations on *Phrynosoma* (cone retina), and for comparison the curves obtained with turtle (cone retina), gecko (rod retina), and the cone portions of the duplex curves for frog and Platy; see text.

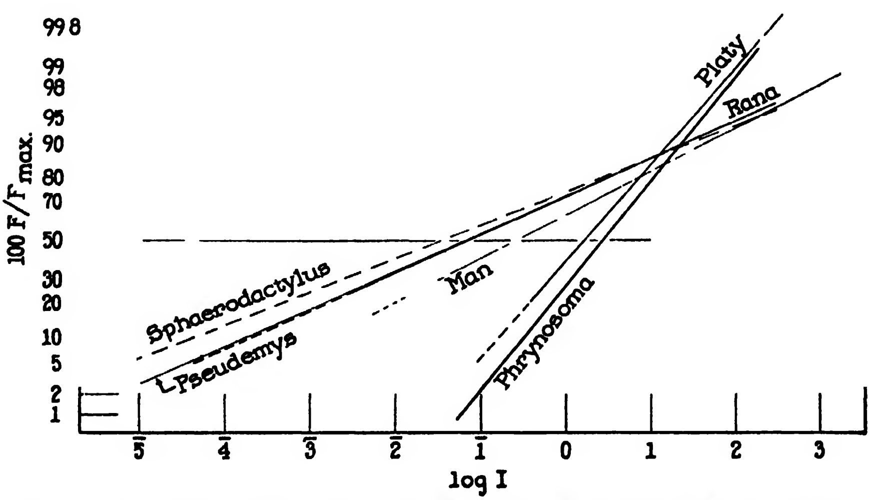

FIG. 3. The curves of Fig. 2, and the cone curve for a human subject, transferred to a probability grid ($F_{max.}$ = 100 in each case), to facilitate certain comparisons; see text.

course another matter. But we also know that $F_{max.}$ is a function of the light time fraction in the flash cycle (*cf.* Crozier, Wolf, and Zerrahn-Wolf, 1937–38 *d*; Crozier and Wolf, 1939–40 *c*; 1940–41 *a*) and that the factors of direct rectilinear proportionality connecting $F_{max.}$ and τ' with the per-

centage dark time are functions of the particular animal. Since we likewise know that the shape constant ($\sigma'_{\log I}$) for the (cone) response contour is independent of the flash cycle light time ratio, the proper basis for the intercomparison of the forms of the various contours is found in their representation on a probability integral grid in which for each case $F_{max.}$ = 100 per cent. This is given in Fig. 3 from the data on various vertebrates already represented in Fig. 2, and in additional data on man (*cf.* Crozier, Wolf, and Zerrahn-Wolf, 1937–38 *b*). The respective values of $F_{max.}$ (for $t_L = t_D$) are: Man, 56.7; *Pseudemys*, 52.6; *Platypoecilius*, 46.1; *Phrynosoma*, 42.4; *Rana*, 40.5; *Sphaerodactylus*, 26.8. It is apparent from a consideration of these and other similar data that there is no formal association among the values of the three parameters of the probability integral which efficiently describes these data. This conclusion is reinforced by the examination of comparable data provided by various arthropods (*cf.* Crozier, Wolf, and Zerrahn-Wolf, 1938–39 *b*). There is thus given one part of the proof that three independent parameters are necessary and sufficient for the description of the flicker response contour,—such as are provided by the three constants of the probability integral, namely the asymptotic maximum $F_{max.}$; the abscissa of inflection τ'; and the standard deviation of the first derivative of the curve, $\sigma_{\log I}$. The complementary portion of the proof is obtained from the results of independently modifying each of these parameters through the experimental control of retinal area, body temperature, and the light time ratio in the flash cycle (*cf.* Crozier and Wolf, 1939–40 *c*).

The data on *Phrynosoma* reinforce considerations respecting the general doctrine of visual duplexity in vertebrates which we have elsewhere mentioned (Crozier, Wolf, and Zerrahn-Wolf, 1938 *a*; Crozier and Wolf, 1938 *a*, 1938–39 *b*, 1939). Purely cone flicker threshold curves of a turtle, the horned toad, and a bird (zebra finch) considered in a succeeding paper, are found to differ more in essential respects than do the (purely rod) curve of *Sphaerodactylus* and the (cone) curve of *Pseudemys*. We are well aware that in entering upon such comparisons it must not be lost sight of that the index responses of each animal used are necessarily peculiar to itself. But under the conditions of these tests the end-points are at least congruous in the sense that they are threshold motor responses. As such they probably have as much essentially in common, among different animals, as do the rod and cone end-points in a given animal with visual duplexity—for example, in man. In any case, therefore, the correlation of histological receptor types with visual discriminative capacity in the matter of flicker cannot be made on the basis of the quantitative properties of the response contours alone.

IV

SUMMARY

The lizard *Phrynosoma*, with purely cone retina, provides a simplex flicker response contour (log critical flash intensity as a function of flash frequency). It is well described as a normal probability integral (F – log I).

The *Phrynosoma* curve differs markedly, in higher slope and in higher median intensity level, from that obtained under the same conditions for the turtle *Pseudemys*, also with entirely cone retina. Other comparisons having a bearing on the duplexity doctrine are discussed.

CITATIONS

Chaffee, E. L., and Sutcliffe, E., 1930, *Am. J. Physiol.*, **95**, 250.

Crozier, W. J., 1935–36, *J. Gen. Physiol.*, **19**, 503.

Crozier, W. J., and Wolf, E., 1938 *a*, *Proc. Nat. Acad. Sc.*, **24**, 538; 1938 *b*, **24**, 542. 1938–39 *a*, *J. Gen. Physiol.*, **22**, 463; 1938–39 *b*, **22**, 555. 1939, *Proc. Nat. Acad. Sc.*, **25**, 171. 1939–40 *a*, *J. Gen. Physiol.*, **23**, 143; 1939–40 *b*, **23**, 229; 1939–40 *c*, **23**, 531; 1939–40 *d*, **23**, 667; 1939–40 *e*, **23**, 677; 1940–41 *a*, **24**, in press; 1940–41 *b*, in press.

Crozier, W. J., Wolf, E., and Zerrahn-Wolf, G., 1936–37 *a*, *J. Gen. Physiol.*, **20**, 211; 1936–37 *b*, **20**, 393. 1937, *Proc. Nat. Acad. Sc.*, **23**, 516. 1937–38 *a*, *J. Gen. Physiol.*, **21**, 17; 1937–38 *b*, **21**, 203; 1937–38 *c*, **21**, 223; 1937–38 *d*, **21**, 313. 1938 *a*, *Proc. Nat. Acad. Sc.*, **24**, 125; 1938 *b*, **24**, 221. 1938–39 *a*, *J. Gen. Physiol.*, **22**, 311; 1938–39 *b*, **22**, 451.

Detwiler, S. R., and Laurens, H., 1920, *J. Comp. Neurol.*, **32**, 347.

Ehrenhardt, H., 1937, *Z. vergleich. Physiol.*, **24**, 258.

Garten, S., 1907, Die Veränderungen der Netzhaut durch Licht, in Graefe, A., and Saemisch, T., Handbuch der gesamten Augenheilkunde, Berlin, Julius Springer, **3**, pt. 1, chapter 12, Anhang, 2nd edition.

Keeler, C. E., 1930, *J. Morph. Physiol.*, **50**, 193.

Loeb, J., 1891, *Arch. ges. Physiol.*, **49**, 175; 1907, **116**, 368.

Loeb, J., 1918, Forced movements, tropisms, and animal conduct, Philadelphia and London, Lippincott.

Menner, E., 1929, *Z. vergleich. Physiol.*, **8**, 1. 1938, *Zool. Jahrb.* (*Abt. allg. Zool. Physiol.*), **58**, 481.

Meservey, A. B., and Chaffee, E. L., 1927, *J. Opt. Soc. America*, **15**, 311.

Ohm, J., 1931, *Arch. Ophth.*, Leipzig, **126**, 547.

Rochon-Duvigneaud, A., 1917, *Ann. ocul.*, Brussels, 1917, 1.

Schlieper, C., 1927, *Z. vergleich. Physiol.*, **6**, 453.

Trendelenburg, W., and Kühn, A., 1908, *Arch. Anat. u. Physiol.*, **2**, 160.

Verrier, M. L., 1935, *Bull. biol. France et Belgique*, suppl. **20**.

Walls, G. L., 1934, *Am. J. Ophth.*, St. Louis, **17**, 892.

Wolf, E., and Zerrahn-Wolf, G., 1935–36, *J. Gen. Physiol.*, **19**, 495.

ISOLATION, CRYSTALLIZATION, AND PROPERTIES OF PEPSIN INHIBITOR

By ROGER M. HERRIOTT

(From the Laboratories of The Rockefeller Institute for Medical Research, Princeton, New Jersey)

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When a solution of pepsinogen is acidified to pH 1.0–5.0, one or more reactions take place producing pepsin and certain polypeptides. It was previously noted by the writer that one of these polypeptides has a powerful inhibiting action on pepsin at pH 5.0-6.0 (1). The present paper describes the isolation, crystallization, and properties of this inhibitor of pepsin.

The decrease in milk clotting activity at pH 5.7 of a standard pepsin solution is used as a measure of inhibitor activity.

Preparation of the inhibitor consists of activation of the pepsinogen at pH 1.0-2.0 for a very short time followed by rapid alkali inactivation of the pepsin. Precipitation of the denatured pepsin is brought about with trichloracetic acid. The inhibitor remains dissolved in the trichloracetic acid filtrate. The total nitrogen in the trichloracetic acid filtrate is about 15 per cent of the original pepsinogen nitrogen; half of this non-protein nitrogen is inhibitor nitrogen. Separation of the inhibitor from the inert polypeptides has been accomplished by repeated fractional precipitation first with tungstic acid at pH 1.0-2.0 and second by magnesium sulfate in the presence of trichloracetate ion at about pH 3.0. When fractionation has brought the specific inhibiting activity, *i.e.* the inhibiting activity per milligram nitrogen $[\text{I.U.}]_{\text{mg. N}}$ to above 60 per cent of the value of the pure inhibitor, the preparation may be crystallized. Half saturated ammonium sulfate, pH 5.0, room temperature, and 3.0–5.0 mg. of inhibitor nitrogen per ml. are the conditions required for crystallization. The material first precipitates as clear spheroids which on standing form rosettes of fine needles, as may be seen in Fig. 1.

Fractional recrystallization and solubility experiments indicate the presence of not more than 20-25 per cent impurity in the material of highest specific activity.

The inhibitor is destroyed by pepsin between pH 2.0-5.0, with a rate maximum near pH 4.0.

The reversible combination of pepsin with the inhibitor follows quantitatively the simple mass law equation arranged for a similar reaction by Northrop (2).

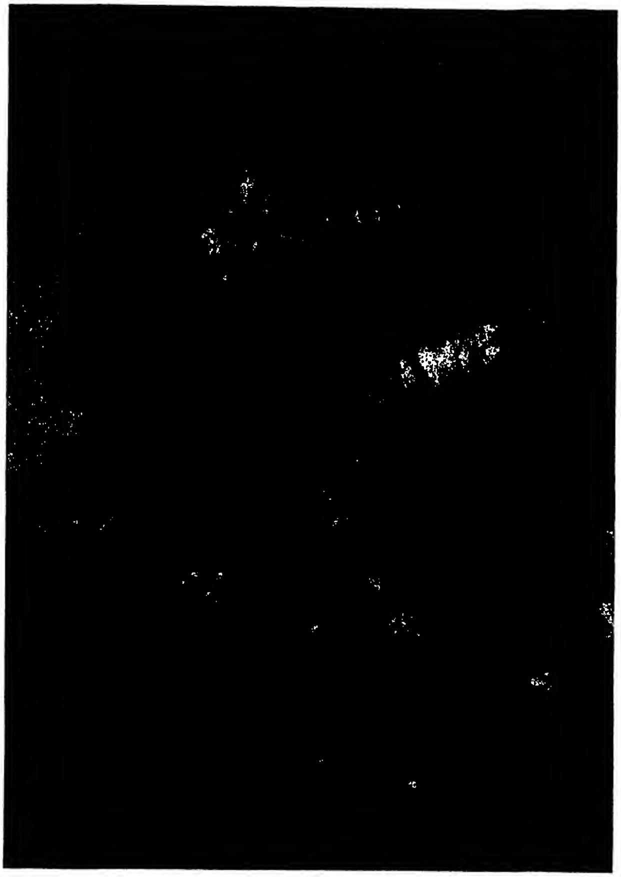

Fig. 1. Crystalline inhibitor of pepsin

The proteolytic action of pepsin as well as the milk clotting action is inhibited at pH 5.7. Dissociation of the pepsin inhibitor complex prevents tests at a more acid pH. There was no demonstrable effect of the inhibitor on crystalline trypsin as measured by the digestion of hemoglobin at pH 7.0–8.0, nor the milk clotting action of crystalline chymotrypsin or commercial rennet at pH 5.7. The crystalline trypsin inhibitor (3) had no effect on the milk clotting action of pepsin. This indicates a high degree of specificity among the inhibitors and is additional proof that the enzyme, rennet, is different from pepsin. An interesting result was obtained when

pepsin from different species was tested with swine pepsin inhibitor. Bovine pepsin was inhibited to the same degree as swine pepsin but chicken pepsin was not inhibited at all. On the other hand, crude inhibitor solution prepared from chicken pepsinogen inhibited both swine and bovine pepsin but had no effect on the chicken pepsin.

Certain chemical and physical properties have been determined, such as the isoelectric point, optical rotation, elementary analysis, amino nitrogen, and rate of diffusion. A few amino acids making up the inhibitor have been roughly estimated as well as the number of peptide linkages. The indications are that the inhibitor has basic groups exposed since it is precipitated by many reagents used to precipitate basic substances, namely, tungstic, phosphotungstic, flavianic, picric, and picrolonic acids. The main basic amino acid is probably arginine. The molecular weight, as determined by diffusion and the tyrosine content and combining equivalence with pepsin lies somewhere between 4,000 and 10,000.

EXPERIMENTAL RESULTS

Preparation of Crystalline Inhibitor

Preparation of Crude Inhibitor Solution from Pepsinogen.—Purified swine pepsinogen as previously described (1) was first dialyzed; adjusted to 2 mg. P.N./ml. and titrated to pH 2.0, temperature = 20–25°C. for 1 minute, then brought to pH 11.0–12.0 with 5 N sodium hydroxide where it was allowed to remain for 5 minutes. Acidification to pH 2.0 was then brought about by the addition of 3.0 normal trichloracetic acid. These reagents were all added in fairly concentrated form to prevent too great a dilution and the amount required determined on an aliquot so that all solutions could be mixed rapidly. After standing about 20 minutes the precipitate was filtered off with suction and washed with 2.5 per cent trichloracetic acid. The filtrate and washings containing the inhibitor were combined and, as may be seen, solution No. 2 of Table I (analysis of an actual preparation) contains about 0.11 mg. inhibitor units $[\text{I.U.}]_{\text{ml.}}$ and 0.3 mg. total nitrogen per ml. Thus the specific $[\text{I.U.}]_{\text{mg. N}}$ is 0.3. From this it follows that one gets about 0.06 [I.U.] per milligram of original pepsinogen nitrogen.

Fractionation Procedures

Tungstic Acid.—The inhibitor is precipitated from the trichloracetic acid filtrate as follows: 0.2 ml. of 5 per cent sodium tungstate (0.16 M) is added for every 100 ml. of filtrate, the precipitate centrifuged or filtered, and discarded. To the supernatant is added 5 ml. of 5 per cent sodium tungstate per 100 ml. supernatant. This residue containing about 75 per cent of the activity is dissolved by titrating slowly until pink to phenolphthalein. The tungstate is precipitated by addition of an excess of barium chloride and the tungstate centrifuged off followed by removal of excess barium ion by acidification with dilute sulfuric acid and addition of sodium sulfate until no further precipitate of barium sulfate appears.

Magnesium Sulfate-Trichloracetate Ion.—After filtering off the barium sulfate and

TABLE I

Preparation of Pepsin Inhibitor

Procedures and materials	No.	Quantity	[I.U.]ml.	Total [I.U.]	N/ml.	[I.U.]/N
		ml. or gm.			*mg.*	
Dialyzed freshly prepared pepsinogen.........	1	1032			5.2	
Solution No. 1 + 1 liter water + 80 ml. N/1 hydrochloric acid for 1 min. at 25°C. then + 150 ml. N/1 sodium hydroxide, pH = 11; allowed to stand 3 min. followed by 50 ml. 3 N trichloracetic acid, pH = 2; left 20 min., filtered and residue washed with 300 ml. 2.5 per cent trichloracetic acid. Residue discarded. Filtrate and washings............	2	2665	0.11	290	0.33	0.3
No. 2 + 5 ml. of 5 per cent sodium tungstate solution, stirred + 5 gm. Filter-Cel, filtered and to the filtrate was added 150 ml. 5 per cent sodium tungstate, filtered with the aid of 10 gm. of Filter-Cel. Precipitate....	3P					
Filtrate.......	3F	2800	0.0032	9	0.075	0.043
No. 3P + 500 ml. water + 40 ml. N/1 sodium hydroxide to pH 9 (pink to phenolphthalein) then + 75 ml. M/1 barium chloride, let stand 10 min., filtered and washed precipitate twice with water. Filtrate + 75 ml. M/1 sodium sulfate + 0.5 N sulfuric acid to pH 3.5; filtered. Filtrate.......	4	725	0.3	220	0.74	0.4
No. 4 + 1450 ml. of the magnesium-trichloracetate solution (2750 ml. saturated magnesium sulfate + 200 ml. 3 N trichloracetic acid + 30 ml. 18 N sodium hydroxide) let stand 48 hrs. and filtered with the aid of 15 gm. Hyflo Super-Cel Precipitate....	5P					
Filtrate.......	5F	2200	0.015	33	0.087	0.17
Inhibitor dissolved out and separated from the Super-Cel Solution......	6	158	1.15	182	2.0	0.57
No. 6 diluted to 1800 ml. with water then + 6 ml. 5 N hydrochloric acid to pH 2.0 followed by 90 ml. 5 per cent sodium tungstate with stirring. Filtered. Residue dissolved in water + N/1 sodium hydroxide until solution is pH 9.0; then + 20 ml. M/1 barium chloride, centrifuged and residue washed once with 10 ml. water. To the combined filtrate and washings 20 ml. M/1 sodium sulfate and sulfuric acid to pH 3.0 was added followed by filtration. Residue washed on funnel. Filtrate.......	7	134				
No. 7 + 200 ml. of the magnesium-trichloracetate solution (1.5 vol.) + 1 gm. of Hyflo Super-Cel, filtered Filtrate.......	8F	330	0.082	27	0.22	0.37
Precipitate....	8P					

TABLE I—*Concluded*

Procedures and materials	No.	Quantity	[I.U.]/ml.	Total [I.U.]	N/ml.	[I.U.]/N
		ml. or gm.			*mg.*	
8P + water, filtered and residue washed......	9	115	1.13	130	1.8	0.63
No. 9 diluted to 240 ml. with water then + 5 ml. 5 N hydrochloric acid to pH 2.0 and + 50 ml. 5 per cent sodium tungstate; centrifuged. Residue + 15 ml. 0.5 N sodium hydroxide + 1.5 ml. 5 N sodium hydroxide to pH 9.0 then + 10 ml. M/1 barium chloride let stand 0.5 hr., centrifuged and residue washed with 5 ml. water. Washings + supernatant + 6 ml. M/1 sodium sulfate + 0.5 N sulfuric acid to pH 5.0, cooled to 5°C., centrifuged, supernatant...........................	10	52	1.95	100	3.6	0.54
No. 10 + 1.25 ml. 4 M pH 5.0 acetate + 16 gm. solid ammonium sulfate to 0.5 saturation, stirred at 25°C., solution clear for at least 0.5 hr. Left 20 hrs. at room temperature. Good crystals formed. Aliquot centrifuged. Supernatant...	11					
Crystal precipitate dissolved.................	12					
Inhibitor in mother liquor No. 11 separated from ammonium sulfate by precipitation with an excess of sodium tungstate at pH 2.0 followed by removal of tungstate ion from residue by solution at pH 9.0 then addition of barium chloride and after filtration of barium tungstate, excess barium was removed from the filtrate by addition of excess sodium sulfate Solution......	13		0.05		0.24	0.2
A similar treatment was used to separate the inhibitor from the ammonium sulfate in the solution of the crystals, No. 12.............	14		0.5		0.66	0.76

analyzing for inhibitor activity and nitrogen the inhibitor is next precipitated by the addition of 2 volumes (or if the inhibitor solution contains over 1 mg. nitrogen per ml. 1.5 volumes) of a reagent made up of 200 ml. 3.0 N trichloracetic acid + 30 ml. 18 N sodium hydroxide + 2750 ml. saturated magnesium sulfate. The residue is retained and the filtrate discarded. The residue dissolves readily in water.

These fractionation procedures were applied alternately. After each fractionation step both residue and filtrate were analyzed for inhibitor activity and total nitrogen before discarding either. Fractionation was continued by these two procedures until the activity nitrogen ratio was 0.6 or better.

Crystallization

The fractionation treatment prior to crystallization was a tungstate treatment leaving the inhibitor solution practically salt free. The solution, having approximately 5 mg.

nitrogen per ml. was then titrated to pH 5.0 with sodium acetate or acetic acid and solid ammonium sulfate added with care until the concentration was 0.5 saturated. This requires 31.5 gm. per 100 ml. solution. The solution should be clear after dissolving the ammonium sulfate but after a few minutes a precipitate appears composed of microscopic spheroids and on standing 12–24 hours the spheroids change to rosettes of tiny needles (Fig. 1). Recrystallization may be carried out by solution of the crystals in a small volume of M/10 pH 5.0 acetate followed by addition of an equal volume of saturated ammonium sulfate solution at room temperature.

Tests of Purity

Fractional Recrystallization.—An inhibitor preparation was fractionally recrystallized to see if there was any drift in properties indicating the presence of impurities. The results in Table II show no gross separation or drift indicating an inhibitor of higher activity. The differences are of the same order as the error of the experimental methods.

TABLE II

Fractional Recrystallization of Inhibitor

Fraction	Original material	Specific activity [I.U.]mg. N
	per cent	
First crystal cake	100	0.83
Second crystal cake	33	0.95
Mother liquor of second crystallization	16	0.95
Third crystal cake	13	0.95
Mother liquor of third crystallization	20	0.75

Solubility Curve.—The solubility curve shown in Fig. 2 indicates that the preparation having a specific inhibitory value of 0.9 probably contains not more than 25 per cent impurity.

Experimental Procedure

Fig. 2 represents the solubility of the amorphous form of the inhibitor at 23°C. approached from the supersaturated side of the equilibrium. The technique was for the most part similar to that described elsewhere (4). The solvent used in this experiment is composed of two solutions, first the dissolving solution, M/50 pH 4.0 acetate buffer, and the precipitating solution made up in the following way: to 2750 ml. of saturated magnesium sulfate, specific gravity 1.296, is added 200 ml. of 3.0 N trichloracetic acid and 30 ml. 18 N sodium hydroxide. The complete solvent is made up of 45 parts of the dissolving acetate and 55 parts of the precipitating magnesium sulfate-trichloracetate.

Inactivation of Inhibitor by Pepsin

It was soon found that whereas pepsin free inhibitor solutions are stable for long periods of time at acidities varying from pH 1.0–10.0 in the presence

of pepsin the inhibitor is rapidly inactivated with a pH maximum near pH 3.5, as may be seen in Fig. 3. There is an increase in amino nitrogen, as measured by Van Slyke's gasometric method, amounting to approximately 8 per cent of the total nitrogen or about 5 amino groups per molecule if we assume a likely molecular weight of 5,000 for the inhibitor.

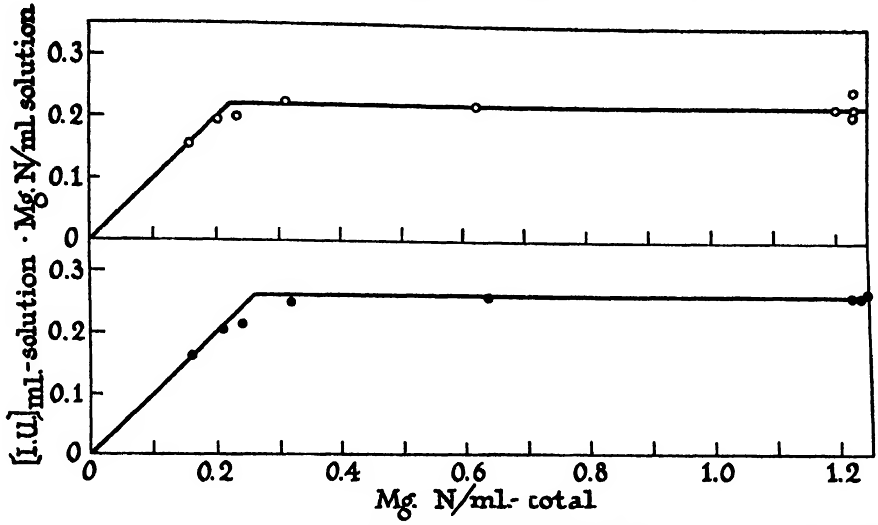

FIG. 2. Amorphous solubility curve of inhibitor in a magnesium sulfate-trichloracetate solution at 23°C.

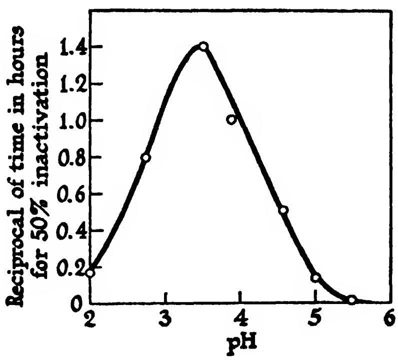

FIG. 3. pH-inactivation curve of inhibitor in the presence of pepsin

These experiments are strong evidence that the inactivation of the inhibitor is in fact a hydrolysis catalyzed by pepsin. In this connection it might be pointed out that the pH maximum at pH 3.5–4.0 shown in Fig. 3 is close to that found by Fruton and Bergmann for the hydrolysis of their synthetic substrates by pepsin (5).

Experimental Procedure

To 2 ml. of a pepsin solution at approximately the desired pH containing 250 rennet units per ml. were added 2 ml. of a crude inhibitor solution containing 250 inhibitor units per ml. and 0.5 ml. of M/10 acetate buffer of the desired pH. The temperature was 35°C. At certain time intervals samples were taken and diluted 0.1/10 in M/10 pH 5.7 acetate. After standing 30 minutes the milk clotting activity was determined and the amount of inhibitor calculated. Curves were plotted and the times for 50 per cent destruction were read off the curves.

For the amino nitrogen analyses a highly purified sample of inhibitor of specific activity = 1.0 was used. To a solution containing 1.6 mg. inhibitor nitrogen at pH 3.5 was added a dialyzed pepsin solution to bring the pepsin protein nitrogen to 0.2 mg. per ml. Immediately after mixing and again in 60 hours samples were analyzed for inhibitor action and Van Slyke amino nitrogen.

Application of the Mass Law to the Combination Reaction of Pepsin and Inhibitor

Some evidence has been obtained to indicate that the combination of pepsin with the inhibitor is a simple reversible dissociation as illustrated in equation I

$$\text{Pepsin} + \text{inhibitor} \rightleftharpoons \text{inhibitor pepsin compound} \tag{I}$$

This type reaction should follow the mass law which in its simplest form is equation II

$$\frac{[\text{Pepsin}] \quad [\text{inhibitor}]}{[\text{Pepsin-inhibitor compound}]} = \text{constant} \tag{II}$$

where the values within brackets are concentrations. In using the simplest form it is assumed that one molecule of pepsin reacts with one molecule of inhibitor.

Equation II cannot be used as such but may be rearranged as was done by Northrop (2) so that it will contain terms that are easily measurable. The equation used in the present work is the same as that used by Northrop with a few minor changes in symbols and is equation III,

$$P_f = \pm\sqrt{\left(\frac{I_t - P_t + K}{2}\right)^2 + KP_t} - \frac{I_t - P_t + K}{2} \tag{III}$$

where P_f = free pepsin; P_t = total pepsin; I_t = total inhibitor expressed in terms of pepsin units; and K = the constant for the equation.

In order to use this equation, the total inhibitor concentration used must be constant and the total pepsin concentration varied. The free or uncombined pepsin is determined and the combined pepsin obtained by the difference between the total and free pepsin. It is also necessary to make

certain assumptions which, as nearly as could be tested, are valid namely, that the pepsin inhibitor compound has no activity and that on addition of the compound into the Klim solution there is no appreciable dissociation due to dilution before the Klim is clotted.

When the amount of total pepsin is plotted against the combined pepsin we get a smooth curve, as seen in Fig. 4, which approaches a limiting value depending on the amount of inhibitor used. Since the above equation

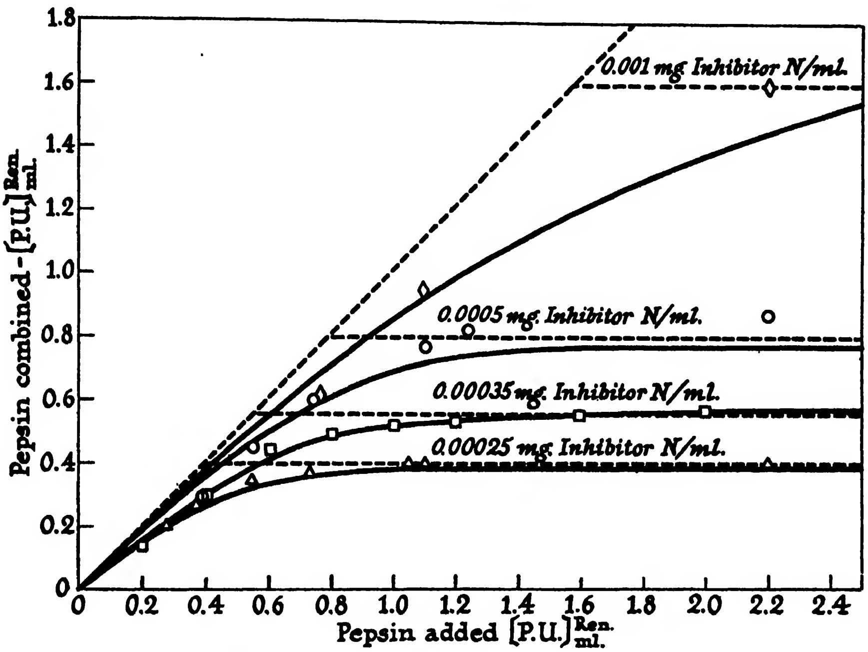

FIG. 4. Effect of increasing amounts of pepsin and inhibitor on the amount of pepsin bound by the inhibitor at pH 5.7. The points are the determined values. The solid lines are the theoretical curves calculated from the mass law as indicated in the text. The broken lines indicate the course if the reaction were stoichiometric.

(III) calls for the inhibitor in terms of pepsin units, this limiting value or maximum value of pepsin to combine with the indicated amount of inhibitor can be substituted for the inhibitor concentration. In other words, the total inhibitor is expressed as that amount of pepsin with which the inhibitor will combine when there is a large excess of pepsin.

In the present instance the pepsin equivalent value for the inhibitor was obtained only from curve I; *i.e.*, 0.00025 mg. inhibitor nitrogen is equivalent to 0.4 pepsin rennet units. The pepsin equivalent values for the inhibitor in curves II, III, and IV were calculated from curve I. This was possible

since the amount of inhibitor nitrogen used in these curves was predetermined.

The solid lines are the calculated curves obtained by calculating back with the equation using an average value of the constant K.

It may be seen in Fig. 4 that the experimental points show a reasonable approach to the calculated curves.

If one takes the figures obtained from the above experiment, namely that 0.00025 mg. inhibitor nitrogen is equivalent to 0.4 rennet units of pepsin or approximately 0.0012 mg. pepsin nitrogen, one may then calculate the molecular weight of the inhibitor. Such a calculation has been performed and it indicates a molecular weight of about 7,000.

TABLE III

Reaction of Inhibitors from Different Species with Different Pepsins

Source of inhibitor	Enzyme	Substrate	pH	Inhibiting action
Swine pepsinogen	Swine pepsin	Klim (milk clotting action)	5.7	+
" "	" "	Denatured pepsin	5.7	+
" "	" "	" hemoglobin	5.7	+
" "	Bovine "	Klim (milk clotting action)	5.7	+
" "	Chicken "	" " " "	5.7	−
Chicken "	Swine "	" " " "	5.7	+
" "	Bovine "	" " " "	5.7	+
" "	Chicken "	" " " "	5.7	−
Bovine trypsin inhibitor	Swine "	" " " "	5.7	−
Swine pepsinogen	Bovine chymotrypsin	" " " "	5.7	−
" "	" rennet	" " " "	5.7	−
" "	" trypsin	Denatured hemoglobin	7.6	−

Experimental Procedure

To a 1 ml. amount of inhibitor solution (the concentration of which is stated in Fig. 4) in a series of tubes was added a 1 ml. of pepsin of various concentrations dissolved in M/10 pH 5.7 acetate. These solutions were left at 35°C. for 30 minutes after which the milk clotting activity was determined in the usual way. The inhibitor had a specific inhibiting value per milligram of nitrogen of 0.9 while the pepsin was a 2 times crystallized Cudahy pepsin preparation of $[\text{P.U.}]^{\text{Hb}}_{\text{mg.P.N.}} = 0.3$ and $[\text{P.U.}]^{\text{Ren.}}_{\text{mg.P.N.}} = 300$.

Comparisons of Inhibitors from Various Sources on Different Enzymes

It was of interest to see whether the pepsin inhibitor had any action on other proteolytic enzymes than pepsin and to see if other inhibitors affected

pepsin. In the experiments, the results of which are summarized in Table III, all concentrations of inhibitor were equal to or greater than that used in the normal pepsin estimation so that one might easily expect to detect any appreciable action of the inhibitor.

The results show that the high degree of specificity usually associated with enzymes also exists among some inhibitors of enzymes. This is shown clearly in the fact that rennet from calves' stomachs is not inhibited whereas the milk clotting activity of bovine pepsin is inhibited to exactly the same degree as swine pepsin. On the other hand, chicken pepsin is not inhibited but the inhibitor prepared from activated chicken pepsinogen inhibits swine and bovine pepsin but not the homologous chicken pepsin.

Chemical and Physical Properties

Some of the chemical and physical properties of the purified inhibitor $[\text{I.U.}]_{\text{mg. N}} = 0.95$, have been collected together in Table IV.

In Table IV *B* are a few amino acid analyses along with certain other analyses and certain values deduced from them. For instance, assuming the molecular weight to be 5,000 there are then 57 atoms of nitrogen per molecule of inhibitor. In the intact inhibitor there are 8 free amino nitrogens while after acid hydrolysis there are 38. It follows therefore that there has been an increase of 30 amino groups but 3–4 of this increase of amino groups was found to be the amide nitrogen which on acid hydrolysis yields ammonia. Therefore there are a possible 26 peptide linkages. There must also be some 19 non-amino nitrogen. The arginine content of 31 per cent represents about 7–8 molecules of arginine per inhibitor. Since 3 of the 4 nitrogens in the arginine molecule are non-amino all of the non-amino nitrogen can be explained by the arginine content. The tyrosine content is so low that a fairly exact molecular weight can be obtained. The analysis by the Folin phenol test after acid hydrolysis of the inhibitor and comparison with a solution of standard tyrosine yields 0.32 per cent which is 1 tyrosine per molecule of a 4400 molecular weight inhibitor or 2 per molecule of 8,800 molecular weight. The tryptophane test of May and Rose is negative.

It seems very likely that the inhibitor has a number of strongly basic groups exposed for it is precipitated from dilute solution practically quantitatively by tungstic, phosphotungstic, flavianic, picric, and picrolonic acids, all of which are supposed to precipitate basic substances. The relatively high content of arginine would tend to account for this.

Experimental Methods

Pepsin Milk Clotting Activity Measurement.—This measurement was carried out exactly as described in the experimental methods of a previous paper (1). The rennet unit of pepsin activity is also defined.

Inhibitor Activity Measurements.—*A. Pepsin Control.*—1 ml. of a solution of pepsin containing 1.0 rennet units, 1.0 [R.U.] in M/10 pH 5.7 acetate was added to 1 ml. of M/10 pH 5.7 acetate buffer; left 10 minutes at 35°C. 0.5 ml. of this solution is then pipetted into 5 ml. of 20 per cent Klim in M/10 pH 5.0 acetate at 35°C. and the time of clotting noted. It should be very close to 2 minutes.

TABLE IV

Chemical and Physical Properties

Property	Method of analysis	Per cent of moisture free material
C		48.07
H		8.07
N	Dumas	16.65
N	Kjeldahl	16.7
Ash		0.5
$[\alpha]_{D}^{\text{pH } 7}$ gm.		−104°
Diffusion constant	Northrop and Anson	0.09 cm.²/day
I. E. P.	Cataphoresis of collodion particles	pH 3.7
Molecular weight	Diffusion	8,000
Molecular weight	Tyrosine content	$(5,000)_n$

		Per cent of total nitrogen	No./molecule*
Primary amino N	Van Slyke gasometric	18	8–9
Amide N	Alkaline distillation after acid hydrolysis	6.5	3
Non-amino N	Difference between total N and NH_2N after acid hydrolysis	23	19
Peptide linkages	Difference between total NH_2-N after hydrolysis and original NH_2-N plus amide N		27
Arginine	Sakaguchi	31	8
Tryptophane	May and Rose	0	0
Tyrosine	Folin phenol	0.4	1

* Assuming a molecular weight of 5,000.

B. Inhibitor.—1 ml. of the same pepsin solution as used in *A* is added to 1 ml. of a dilute (0.0001–0.0003 inhibitor units, [I.U.] per ml.) solution of inhibitor in M/10 pH 5.7 acetate and placed at 35°C. for 10 minutes after which 0.5 ml. is put into Klim and the time required for clotting noted.

C. Calculation of Inhibitor Activity and the Units.—The per cent inhibited is obtained by substituting the two clotting time values in the formula

$$\text{Per cent inhibited} = 100\left[1 - \left(\frac{\text{clotting time of inhibited solution}}{\text{clotting time of control solution}}\right)\right]$$

Having the per cent inhibited, one reads off directly from the curve in Fig. 5 the inhibitor units [I.U.] or the equivalent amount of pure inhibitor nitrogen. The unit inhibitor activity [I.U.] is the inhibiting activity of 1 mg. of N of the purest inhibitor such as that used in obtaining the curve in Fig. 5 which was crystalline and nearly solubility pure. Expressing it in another way, 2×10^{-4} [I.U.] will cause 50 per cent inhibition of the standard pepsin solution when treated as described above. This may be seen by examining Fig. 5. The specific inhibiting activity is merely the inhibiting activity per milligram of nitrogen $[\text{I.U.}]_{\text{mg.N}}$. When the inhibitor is pure the $[\text{I.U.}]_{\text{mg.N}}$ will, of course, then be 1.0.

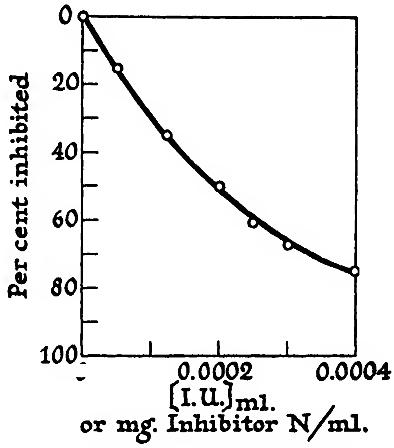

FIG. 5. Inhibitor-activity calibration curve

pH.—Unless otherwise stated all pH determinations were carried out with the aid of Clark and Lubs indicators. The pH values given are those of standard buffer solutions giving the same color with a proportional amount of indicator.

Nitrogen.—Nitrogen estimation was by the micro-Kjeldahl as previously described.

Pepsin.—The pepsin used in the estimation of inhibitor was a glycerinated 2 times crystalline Cudahy preparation having 300 rennet units per milligram protein nitrogen.

Pepsinogen.—The pepsinogen used to prepare the inhibitor was prepared exactly as previously described (1).

SUMMARY

A method has been described for the isolation and crystallization of swine pepsin inhibitor from swine pepsinogen.

Solubility experiments and fractional recrystallization show no drift in specific activity.

The reversible combination of pepsin with the inhibitor was found to obey the mass law.

The inhibitor is quite specific, failing to act on other proteolytic and milk clotting enzymes. The inhibitor is destroyed by pepsin at pH 3.5.

Chemical and physical studies indicate that the inhibitor is a polypeptide of approximately 5,000 molecular weight with an isoelectric point at pH 3.7. It contains arginine, tyrosine, but no tryptophane and has basic groups in its structure.

REFERENCES

1. Herriott, R. M., *J. Gen. Physiol.*, 1938, **21**, 537.
2. Northrop, J. H., *Harvey Lectures*, 1925–26, **21**, 36.
3. Kunitz, M., and Northrop, J. H., *J. Gen. Physiol.*, 1936, **19**, 991.
4. Northrop, J. H., *J. Gen. Physiol.*, 1930, **13**, 739.
   Herriott, R. M., Desreux, V., and Northrop, J. H., *J. Gen. Physiol.*, 1940, **24**, 213.
5. Fruton, J. S., and Bergmann, M., *J. Biol. Chem.*, 1939, **127**, 627.

CHEMICAL PACEMAKERS

III. Activation Energies of Some Rate-Limiting Components of Respiratory Systems*

By ZAREH HADIDIAN and HUDSON HOAGLAND

(*From the Physiological Laboratory, Clark University, Worcester*)

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INTRODUCTION

In a previous paper Hadidian and Hoagland (1939–40) studied activation energies, μ values as calculated from the Arrhenius equation,[1] for the two major components of the crude beef heart extract obtained by the method of Stotz and Hastings (1937). In this system succinic acid loses hydrogen in the presence of the extract's succino-dehydrogenase and becomes fumaric acid. The hydrogen then combines with oxygen in the presence of the extract's cytochrome-cytochrome oxidase. The consumption of oxygen in Warburg vessels can thus serve as a measure of the total reaction's velocity.

Hadidian and Hoagland found that (1) the respiratory enzyme system extracted from the beef heart, and presumably containing two major enzyme components, yielded a μ value of 11,200 $\pm$ 200 calories; (2) this μ value shifts abruptly to 16,000 $\pm$ 200 calories when the enzyme system is poisoned with a *critical* amount of NaCN, thus suggesting that the former value is characteristic of the dehydrogenase activity and the latter of oxidase activity,—since cyanide, by reducing the availability of the oxidase, would, at a critical concentration, make this the limiting slow step

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[1] The Arrhenius equation, $V = Ze^{-\mu/RT}$, describes the speed of a variety of chemical reactions as a function of temperature, where V is chemical velocity, e is the base of natural logarithms, T is the absolute temperature, Z is a constant, R is the gas constant, equal to 1.99 or 2 cal./mol, and μ is the critical thermal increment or energy of activation; *i.e.*, the amount of energy per mol above the average energy of the system required to render the particular molecules reactive. Taking logarithms on both sides of the equation, we obtain, $\log V = C - \mu/2.3\ RT$, and, if the data fit the equation, a plot of $\log V$ against $1/T$ should give a straight line, with intercept C and negative slope $\mu/4.6$. From the slope of the line the μ value in calories per mol may be calculated.

or chemical pacemaker in the chain; (3) this view is further confirmed by the fact that the μ value shifts back to 11,200 calories if a *critical* amount of selenite, shown by Stotz and Hastings to be a specific poison for the dehydrogenase, is added to the enzyme system already poisoned with sufficient cyanide to yield a μ of 16,000 calories. It was thought desirable to study further the various components involved in this reaction system to determine whether these same activation energies, or μ values, could be obtained from the isolated components. If 11,200 and 16,000 calories are respectively characteristic *per se* of succino-dehydrogenase and cytochrome-cytochrome oxidase, then we would expect to obtain these same values in the study of the two components independently. If, on the other hand, these values depend on the reactions (*i.e.*, all the reactants involved in a given step in the reaction), then different values may be obtained in the study of the isolated component enzymes reacting under varying conditions. This latter view is to be expected since the energy of activation refers to energy relationships between particular linkages of enzyme and substrate and these may vary not only from one substrate to another, but different parts of the enzyme molecule may also be active under varying physical and chemical conditions. In our previous paper (Hadidian and Hoagland, 1939–40) we were careful, for example, to point out that the μ value of 16,000 calories obtained under the conditions of our experiments was not necessarily always to be found associated with cytochrome-cytochrome oxidase. The fact that the respective values of 11,200 and 16,000 calories obtained from the enzyme extract agree with values obtained from experiments *in vivo* was regarded as especially significant. The μ value *may be the same* for a given enzyme in the presence of a variety of substrates, as Gould and Sizer (1938) have shown; it may also be independent of pH and other variables over a wide range (Sizer, 1937), thus indicating that the same enzyme *processes* are involved in the splitting of essentially identical substrate linkages from one substrate to another. The temperature method of analysis may thus serve to help unravel the problem of enzyme specificity, both with regard to substrate specificity and to specificity of its own active centers.

To extend the study the following experiments were undertaken: (1) temperature studies of the oxidation of *p*-phenylenediamene catalyzed by the beef heart extract, a reaction presumably involving only the oxidase and not the dehydrogenase component of the system; (2) temperature studies of the succinate oxidation by pyrophosphate-poisoned enzyme; and (3) temperature studies of the succinate oxidation when a dye was substituted for the cytochrome-cytochrome oxidase component after this component had been inactivated by cyanide.

To amplify the reasons for undertaking these three plans of investigation it may be pointed out that the oxidation of *p*-phenylenediamine involves the oxidase component but not the dehydrogenase component, thus affording an opportunity for the independent study of the cytochrome-cytochrome oxidase activity. Pyrophosphate, according to Stotz and Hastings (1937), does not inhibit the dehydrogenase activity, but 6×10^{-5} M of it does inhibit to about 38 per cent the oxidase activity. Thus we might find that the pyrophosphate-partially-poisoned enzyme would yield the same result as the cyanide-partially-poisoned enzyme (*i.e.*, 16,000 calories), since both might presumably make the oxidase step the slow link or pacemaker. Addition of sufficient cyanide to the enzyme system stops the oxidation of succinate completely by poisoning the oxidase. If to this system a reversible oxidation-reduction dye with the proper potential is added as substitute for the oxidase, the oxidation of succinate is restored (Stotz and Hastings, 1937). By suitable choice of dye and of its concentration this reaction may be made to serve as a measure of the activity of the succino-dehydrogenase component.

Procedure

Methods employed in the preparation of the enzyme, the measurement of oxygen consumption, the measurement of reaction velocities, and the calculation of μ values are described in our previous paper (Hadidian and Hoagland, 1939–40).

In all the *p*-phenylenediamine experiments fresh solutions of the *p*-phenylenediamine were prepared for each reaction. It was noted that addition of high concentrations of pyrophosphate to the reaction mixture caused a considerable rise in the pH of the resulting mixture. In all of these experiments the reaction mixture was buffered to pH 7.4 by the addition of KH_2PO_4. When high concentrations of cyanide were used, the pH was adjusted in a similar manner.

Stotz and Hastings (1937) found cresyl blue to be the most efficient dye in replacing the oxidase component. We found experimentally that a mixture containing 0.5 ml. enzyme, 1.0×10^{-5} M cyanide, and 5.0×10^{-6} M cresyl blue in an atmosphere of pure oxygen gave oxygen consumption most nearly approximating that of the unpoisoned, freshly extracted, system.

Our reaction velocities were measured along the approximately linear portions of the reaction curves immediately following the "initial lag." All experiments were repeated at least once and the results found to check within the limits of experimental error.

RESULTS

Oxidation of p-Phenylenediamine

According to Stotz *et al.* (1938) the oxidation of *p*-phenylenediamine involves not only cytochrome *c*-cytochrome oxidase but also the autoxidizable relatively non-cyanide-sensitive cytochrome *b*. In the preparation they

used, about 20 per cent of the oxidation was apparently due to this latter factor functioning concurrently with cytochrome *c*. If such were the case with the preparations used in our experiments, then the Arrhenius equation plot of temperature and velocity of this reaction would be expected to yield not a straight line but a curve concave upwards (Crozier, 1924–25). The fact that our temperature curves are rectilinear, as will be seen, suggests that if both cytochromes are appreciably involved they act sequentially and not concurrently unless the μ values are identical or nearly so. Our linear Arrhenius plots thus indicate either that (*a*) the two cytochrome steps are sequential or (*b*) they act concurrently with activation energies which are of the same order of magnitude. We have, as yet, no satisfactory evidence to enable us to resolve these alternatives.

Fig. 1 shows the oxygen consumption curves at different temperatures for the oxidation of *p*-phenylenediamine. The same enzyme preparation was used on 2 consecutive days. Change in the activity of the enzyme was slight, so that the results of 2 days' experiments could be plotted together. Fig. 2 shows the Arrhenius equation plot obtained from the data of Fig. 1. It is a straight line with a μ value of 9,500. If the concentration of *p*-phenylenediamine is reduced to 1/10 of the concentration used in these experiments, the velocity of the reaction is reduced by more than 60 per cent but the μ value remains the same. It is clearly not 16,000 calories which we had previously obtained when the oxidase component was the slow step in a sequence involving succinate and succino-dehydrogenase.

Enzyme Poisoned with Pyrophosphate

According to Stotz and Hastings (1937) addition of 6.0×10^{-5} M of pyrophosphate does not inhibit the dehydrogenase component, but does inhibit, by 38 per cent, the oxidase component as measured by *p*-phenylenediamine oxidation. It was found that addition of this concentration of pyrophosphate directly to the reaction mixture containing 1.5 ml. of M/15 phosphate buffer (pH 7.4) causes a considerable rise in the pH of the resulting mixture. If the mixture is buffered to pH 7.4 by the addition of KH_2PO_4, there is no inhibition of the *p*-phenylenediamine oxidation by concentrations of pyrophosphate as high as 6.0×10^{-5} M, yet lower concentrations than this at pH 7.4 may cause 90 per cent inhibition in the oxidation of succinate by the enzyme extracts (Fig. 3). The dehydrogenase has been shown not to be poisoned by pyrophosphate (Stotz and Hastings, 1937) and the lack of inhibition of the *p*-phenylenediamine reaction indicates that pyrophosphate does not poison the oxidase component. We

thus have evidence for a step other than the two which involve succinodehydrogenase and cytochrome *c*-cytochrome oxidase in the oxidation of succinate which is acted upon by pyrophosphate. This step apparently is

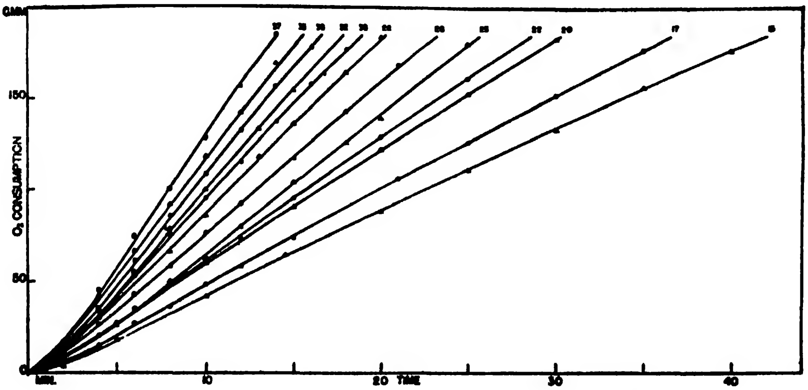

FIG. 1. Oxygen consumption curves for oxidation of *p*-phenylenediamine at different temperatures. 1×10^{-4} M *p*-phenylenediamine and 0.5 ml. enzyme. O = determinations made the day of the preparation of enzyme. △ = determinations made the following day.

The temperature in °C. is given with each curve.

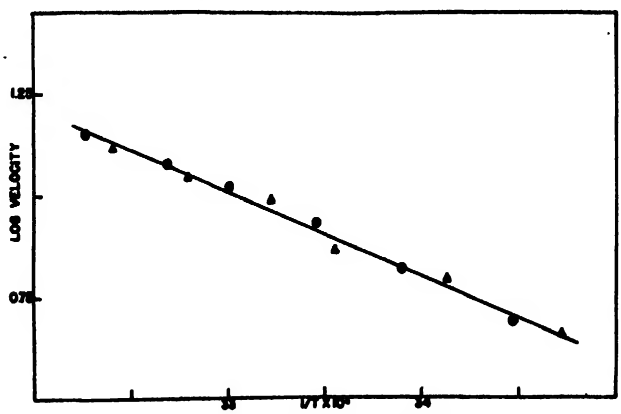

FIG. 2. Arrhenius equation plot of data given in Fig. 1. μ = 9,500

one of the sequence of steps involved in the oxidation of succinate as shown by the high degree of inhibition produced by relatively low concentrations of pyrophosphate.

Temperature studies of pyrophosphate-poisoned reactions yield further support to this view. Fig. 4 shows the Arrhenius equation plot of such

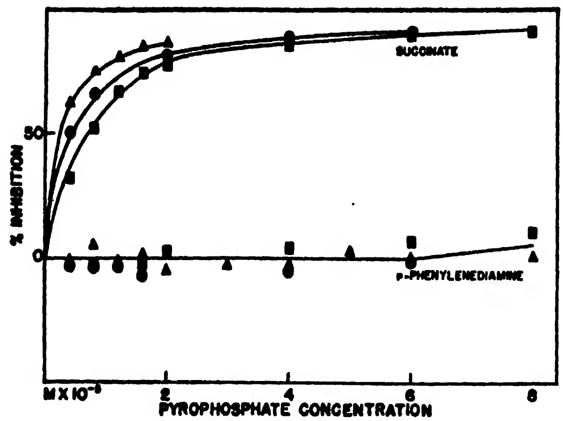

FIG. 3. Effect of varying concentrations of pyrophosphate on the succinate and *p*-phenylenediamine reactions. *p*-phenylenediamine reaction: Temperature 37°C., pH 7.4, 5.0×10^{-5} M *p*-phenylenediamine, 0.5 ml. enzyme. Succinate reaction: Temperature 37°C., pH 7.4, 6.0×10^{-5} M succinate, 0.5 ml. enzyme.

Identical symbols indicate experiments done simultaneously with the same enzyme preparation.

$$\text{Per cent inhibition} = \frac{\text{Velocity of normal reaction} - \text{velocity of poisoned reaction}}{\text{Velocity of normal reaction}}$$

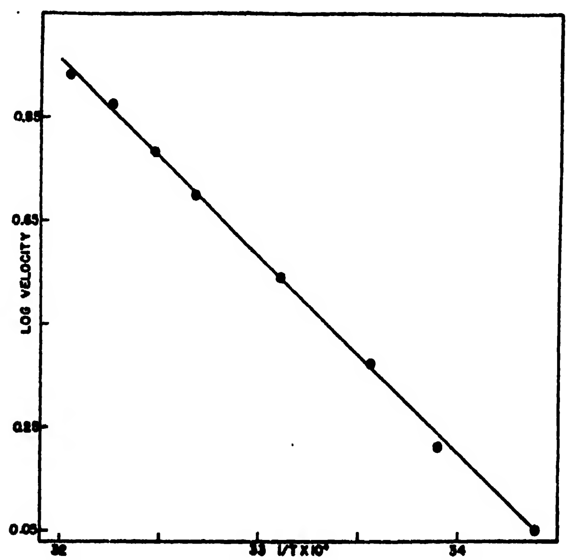

FIG. 4. Arrhenius equation plot of an experiment with pyrophosphate-poisoned enzyme. 6.0×10^{-5} M succinate. 1×10^{-5} M pyrophosphate. 0.5 ml. enzyme. pH 7.4. $\mu = 17{,}500$.

data. A μ of 17,500 calories is obtained which differs from the two other values (11,200 and 16,000) obtained from this system.[2]

Oxidation of Succinate by Enzyme in Which a Dye Is Substituted for the Oxidase

It was thought possible that by poisoning the oxidase component completely and replacing it by a sufficient amount of a suitable dye the normal activity of the enzyme system might be restored. Such a system would afford an opportunity for the study of the succino-dehydrogenase without the cytochrome-cytochrome oxidase component.

It was found experimentally that the addition of 1.0×10^{-5} M cyanide to 0.5 ml. enzyme would stop the oxidation of succinate almost completely (for such a system the O_2 consumption is 2–3 c. mm. for the first 15 minutes). Stotz and Hastings (1937) found cresyl blue to be the most efficient dye in replacing the oxidase component. 5.0×10^{-6} M of this dye added to the completely poisoned system restores the oxygen consumption to normal in the presence of an atmosphere of oxygen (Fig. 5). Addition of this concentration of cresyl blue to the normal reaction mixture causes no inhibition. Varying the concentration of the succinate within large limits does not change the initial velocity of this reaction (Table I). If, however, air is used instead of pure oxygen, the velocity is reduced about 20 per cent.

Fig. 6 shows a series of oxygen consumption curves for this reaction at various temperatures. An Arrhenius equation plot of these data yields a μ value of 18,500 (Fig. 7, curve I). Lowering the concentration of the enzyme from 0.5 to 0.05 ml., while slowing the reaction, does not change the μ value (Fig. 7, curve II), thus indicating that the dehydrogenase-catalyzed step is pacemaker and that its critical increment of 18,500 calories is different in this reaction than that of 11,200 obtained when the chain is that of the "normal" extracted system (Hadidian and Hoagland, 1939–40). Lowering the concentration of the cresyl blue from 5.0×10^{-6} to 5.0×10^{-7} M changes the μ value to 22,000 calories (Fig. 8, curve I). Further lowering of the cresyl blue concentration (by one-half) causes no further significant change in the μ value (Fig. 8, curve II). We thus see that the

[2] By itself this difference in μ would not constitute evidence for another step since it might result from modification of the dehydrogenase by the pyrophosphate. However, taken in conjunction with Stotz and Hastings' evidence that pyrophosphate does not poison the dehydrogenase, and our data shown in Fig. 3, the postulation of an additional step seems necessary.

activation energy of this particular dye-catalyzed step is 22,000 calories and that it is made pacemaker by lowering the dye concentration.

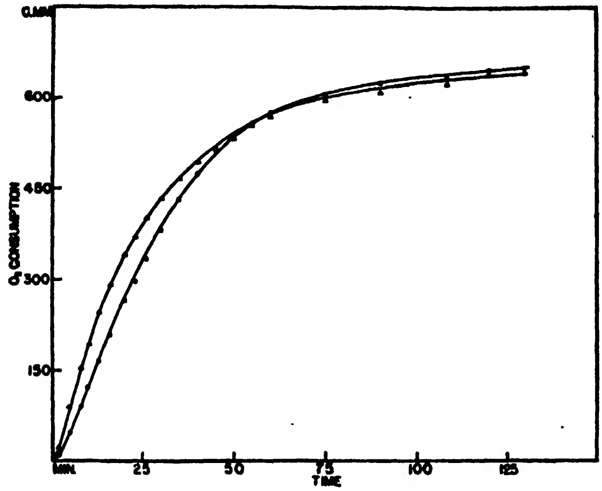

FIG. 5. Oxygen consumption curves for the normal and the dye-substituted reactions. ○ = normal: 6.0 × 1.0⁻⁵ м succinate, 0.5 ml. enzyme. △ = dye-substituted: 5.0 × 10^{-6} м cresyl blue, 1.0 × 10^{-5} м cyanide, 6.0 × 10^{-5} м succinate, 0.5 ml. enzyme.

TABLE I

Temperature 37°C. pH 7.4 0.5 ml. enzyme
Initial velocity taken at 2–5 min. after beginning of reaction

Concentration of succinate м × 10^{-6}/3 cc.	Initial velocity
	c. mm. O_2/min.
15	19.6
12	20.0
10	20.6
8	20.6
6	20.3
4	21.0
2	20.3
1	17.6

Doubly Washed Enzyme

Stotz and Hastings (1937) have reported that by doubling the number of washings a preparation is obtained which, in spite of high oxidase and dehydrogenase activity, showed a lowering of the rate of oxidation of succinate. Such a preparation we found showed a 40 per cent decrease in

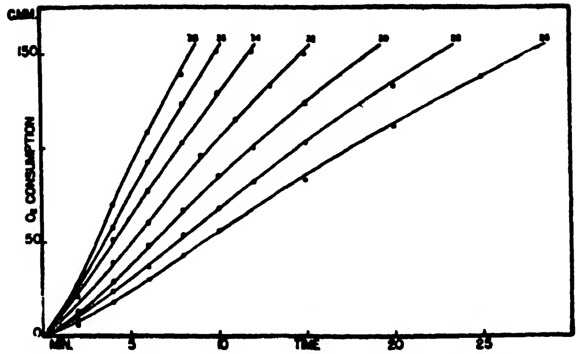

FIG. 6. Oxygen consumption curves for an experiment with dye-substituted enzyme at different temperatures. 5×10^{-6} M cresyl blue, 1.0×10^{-5} M cyanide, 6.0×10^{-5} M succinate, 0.5 ml. enzyme. The temperature in °C. is given with each curve.

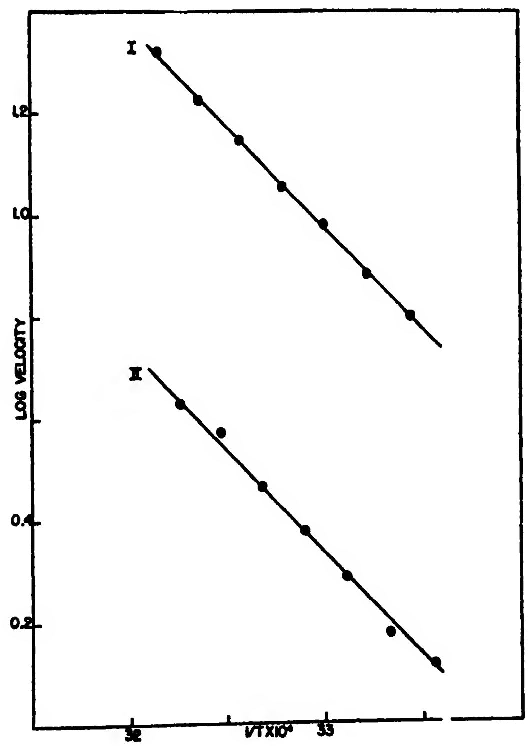

FIG. 7. I. Arrhenius equation plot of data given in Fig. 6. $\mu = 18{,}500$. II. Arrhenius equation plot of an experiment using one-tenth as much enzyme. 6.0×10^{-5} M succinate, 0.1×10^{-5} M cyanide, 0.05 ml. enzyme, 5.0×10^{-6} M cresyl blue. $\mu = 18{,}600$.

the rate of succinate oxidation with the enzyme preparation as obtained after 16 washings (instead of the normal 8), 41 per cent inhibition in the rate of the reaction with 5.0×10^{-6} M cresyl blue substituted for the oxidase component (measure of dehydrogenase activity), and 7 per cent decrease in the rate of oxidation of *p*-phenylenediamine (measure of cytochrome *c*-cytochrome oxidase activity). Decrease in the activity of the enzyme is shown to parallel the decrease in the activity of the dehydrogenase component. The use of this extract might thus yield a μ value characteristic

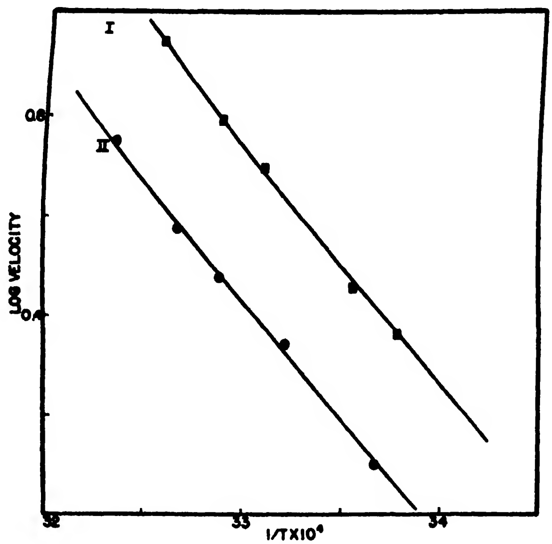

FIG. 8. Arrhenius equation plots of experiments using low concentrations of cresyl blue. I. 6.0×10^{-5} M succinate, 1.0×10^{-5} M cyanide, 0.5 ml. enzyme, 5.0×10^{-7} M cresyl blue. $\mu = 22{,}000$. II. 6.0×10^{-5} M succinate, 1.0×10^{-5} M cyanide, 0.5 ml. enzyme, 2.5×10^{-7} M cresyl blue. $\mu = 22{,}300$.

of succino-dehydrogenase, since more of the dehydrogenase activity is removed by the excessive washing than of the oxidase activity and this should make it the slow step or chemical pacemaker.

The two μ values we have found associated with succino-dehydrogenase are 11,200 calories (Hadidian and Hoagland, 1939–40) when it acts in a sequence with cytochrome *c*-cytochrome oxidase, and 18,500 calories when the dehydrogenase reacts in a sequence in which cresyl blue has been substituted for the cytochrome oxidase after the latter has been inactivated by cyanide. We should thus expect a μ of 11,200 to occur with the doubly washed extract and one of 18,500 calories when the doubly washed enzyme is used with cresyl blue substituted for the oxidase.

Experiments involving the doubly washed enzyme without cyanide and without cresyl blue yield a μ of 11,300 calories, as was expected (Fig. 9, curve II). However, experiments in which the doubly washed enzyme is used with cresyl blue after complete cyanide inhibition of its oxidase also yield 11,300 calories (Fig. 9, curve I) and *not* 18,500. In the first series of experiments the oxygen consumption at a given temperature is approximately linear with time for a considerable period (over 60 minutes at 25°C.). In the second series with cyanide and cresyl blue the curves fall off after about 10 minutes and the rates were calculated from only the first three points at each of the temperatures where the curves of oxygen con-

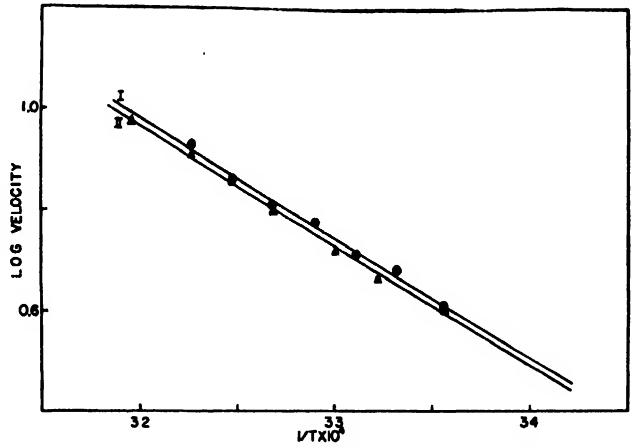

FIG. 9. Arrhenius equation plots of experiments with doubly washed enzyme. I. 5×10^{-6} M cresyl blue substituted for the oxidase component. μ = 11,300 calories. II. Unpoisoned enzyme. μ = 11,300 calories.

sumption and time are linear. The value of 11,300 calories in this second case (Fig. 9, curve I) may be a coincidence. Certainly the data are by no means as reliable as those involved in our other μ plots where the reactions are of zero order over many observations.

Why removal of dehydrogenase activity by washing should yield a different μ from that obtained by reducing the concentration of the enzyme, when in both cases the cytochrome oxidase is completely blocked by cyanide and cresyl blue is substituted in its place, we do not know. If we assume that the data of curve I are reliable, in some unknown way the double washing produces a system in which the μ value for the dehydrogenase reacting with cresyl blue is the same as that encountered for the system reacting with active cytochrome-cytochrome oxidase.

DISCUSSION

In a previous paper (Hadidian and Hoagland, 1939–40) it was concluded that 11,200 calories was the energy of activation associated with succinodehydrogenase activity and 16,000 calories, the energy of activation associated with the cytochrome-cytochrome oxidase activity. In the present study of the two components under different conditions neither of these two values was obtained (except in the case of the doubly washed enzyme). Therefore it is quite evident that these values cannot be associated with

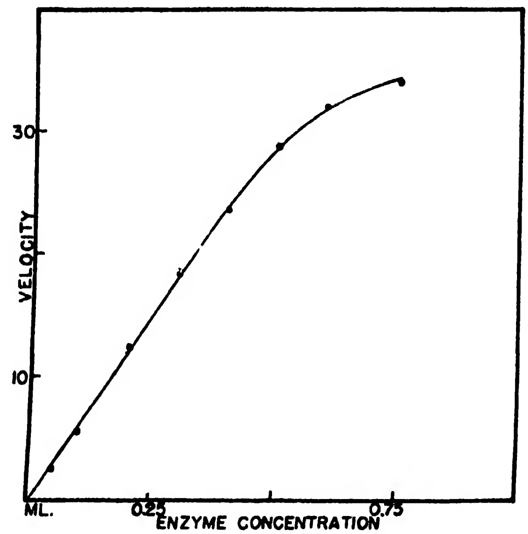

FIG. 10. Effect of varying concentrations of enzyme on the velocity of reaction with dye substituted for the oxidase component. 6.0×10^{-5} M succinate and 5.0×10^{-6} M cresyl blue. The concentration of cyanide to poison the oxidase is varied with that of the enzyme to give total inhibition. Velocity in c. mm. O_2/sec.

these enzymes under all circumstances, but that they characterize the particular step involving them in the reaction as a whole.

The fact that varying the concentration of *p*-phenylenediamine by a factor of 10 does not change the energy of activation indicates that the reduction of cytochrome by *p*-phenylenediamine cannot be the limiting factor in the reaction. This is in agreement with the findings of Stotz *et al.* (1938) that *p*-phenylenediamine reduces cytochrome *c* rapidly.

In the dye-substituted reactions with high concentration of cresyl blue there appears a situation in which the dehydrogenase concentration is the limiting factor. The evidence for this is furnished by the following: (1) the μ value (18,500) does not change with decreasing concentrations of

the enzyme; (2) the velocity of the reaction is a linear function of enzyme concentrations up to 0.5 ml. of enzyme (Fig. 10). With low concentration of the cresyl blue the velocity of the reaction is a linear function of the concentration of the dye. The μ value in this case changes to 22,000 calories.

Since pyrophosphate does not inhibit the dye-substituted reaction (Stotz and Hastings, 1937) nor the oxidation of *p*-phenylenediamine (Fig. 4), it must act on something besides succino-dehydrogenase or cytochrome *c*-cytochrome oxidase. Exactly where it acts to furnish a μ of 17,500 we do not know. It may possibly be on cytochrome *b* or *a* or on some other possible carrier in the sequential chain of reactions.

SUMMARY

1. In a previous paper it was found that 11,200 calories is obtained for the energy of activation in the oxidation of succinate to fumarate in the presence of crude beef heart extract when succino-dehydrogenase was made the limiting factor. 16,000 calories was obtained with this preparation when cytochrome-cytochrome oxidase was made the limiting factor. In the present paper activation energies of the components of this enzyme system are further studied.

2. Oxidation of *p*-phenylenediamine catalyzed by the extract and known not to involve the dehydrogenase component yields Arrhenius equation plots indicating a pacemaker reaction with a μ of 9,500 calories.

3. An activation energy of 17,500 calories is obtained for the oxidation of succinate to fumarate in the presence of the beef heart extract partially poisoned by pyrophosphate. Evidence is presented that this value corresponds to a link in the respiratory chain other than that of succino-dehydrogenase or cytochrome *c*-cytochrome oxidase.

4. Addition of a suitable amount of cresyl blue to a beef heart extract reaction mixture, completely inhibited by cyanide, restores the oxidation of succinate to normal in the presence of pure oxygen. In this system, in which the dye is substituted for the oxidase, when the enzyme extract (dehydrogenase) is made the limiting factor, a μ of 18,500 calories is obtained; when cresyl blue is made the limiting factor, the μ value is 22,000 calories.

5. Results of these experiments indicate that energies of activation are associated not with the enzyme as such, but with the particular reaction steps involving them as catalysts.

CITATIONS

Crozier, W. J., 1924–25, *J. Gen. Physiol.*, **7**, 189.
Gould, B. S., and Sizer, I. W., 1938, *J. Biol. Chem.*, **124**, 269.
Hadidian, Z., and Hoagland, H., 1939–40, *J. Gen. Physiol.*, **23**, 81.
Sizer, I. W., 1937, *J. Cell. and Comp. Physiol.*, **10**, 61.
Stotz, E., and Hastings, A. B., 1937, *J. Biol. Chem.*, **118**, 479.
Stotz, E., Sidwell, A. E., Jr., and Hogness, T. R., 1938, *J. Biol. Chem.*, **124**, 733.

PERMEABILITY OF ERYTHROCYTES TO RADIOACTIVE POTASSIUM

By R. B. DEAN, T. R. NOONAN, L. HAEGE, and W. O. FENN

(From the Department of Physiology, University of Rochester School of Medicine and Dentistry, Rochester, New York)

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It appears to be well established that the red cells are impermeable to cations, but there have nevertheless been some experiments which have demonstrated slow changes in the potassium content. The availability of radioactive isotopes makes possible a re-examination of this question, and some reports based upon this method have already been published.

Cohn and Cohn (1939) showed that radioactive sodium exchanged with sodium in dog erythrocytes *in vivo* so that half the sodium had exchanged in 12 hours. They found that the sodium exchanged as if there were a simple diffusion, taking into account the difference in concentration of sodium between dog cells and plasma. In this laboratory Manery and Bale (1940) have also found evidence of penetration of sodium into dog cells *in vitro*, but their results with rats and rabbits *in vivo* were inconclusive on account of the small amounts of sodium normally present in the cells of these animals.

On the other hand, Hahn, Hevesey, and Rebbe (1939 *a* and *b*) using the radioactive potassium isotope K^{42} found that only about 3 per cent of the potassium in red cells of the frog or rabbit exchanged with the plasma. Joseph, Cohn, and Greenberg (1938 and 1939) working with the rat measured activities in whole blood only, but their data are consistent with a fall in plasma radioactivity followed by a rise in corpuscular activity. Even so they find quite low penetration of radioactive potassium into the red cells. Eisenmann, Ott, Smith, and Winkler (1940) working with human red cells reported no exchange of potassium or sodium. In view of the extremely low sodium content of human cells, it is doubtful whether penetration of sodium could be measured by a radioactive method in this material.

A preliminary abstract of our experiments with radioactive potassium and red cells has already been published (Noonan, Fenn, and Haege, 1940). In this paper they are presented in greater detail.

Methods

Radioactive potassium chloride was prepared by bombarding 100-200 mg. of pure crystals of KCl with a neutron beam of 4.5 m.e.v. for 3 to 4 hours. The crystals were then dissolved in a little water, and the potassium was precipitated as $KClO_4$ by the addition of a saturated solution of NH_4ClO_4. Powdered MnO_2 was added and the mixture was filtered in a porous crucible. The precipitate was washed with alcohol followed by ether and dried. The crucible was then ignited either in a muffle furnace at 500°C. overnight or in a quartz crucible over a Bunsen burner for 1 hour which was found to be sufficient time to reduce the $KClO_4$ to KCl. The crucible was then cooled and weighed. The KCl was dissolved out in a little hot water, transferred to a volumetric flask, and made up to 10 ml. The crucible was dried and weighed again and the quantity of KCl in solution was calculated from the loss in weight. This procedure insured that radioactive sodium which might have contaminated the potassium as well as any activated chlorine was eliminated. There was also sufficient time for radioactive chlorine to disintegrate before the potassium was counted. The original potassium solution was diluted 1/500 for counting and was counted at least every 4 hours while counting of the experimental samples was in progress. Background counts were taken with the same frequency.

In the *in vivo* experiments a portion of the KCl solution (which was approximately isotonic) was injected directly into the animal, intraperitoneally or subcutaneously without anesthesia. Blood from the rabbit was taken from the ear vein into a beaker containing a dry mixture of sodium and ammonium oxalate. It was centrifuged at once. The plasma was drawn off and measured in a graduated centrifuge tube. The cells were washed once with saline (0.85 per cent NaCl) and centrifuged in a graduated centrifuge tube. The volume of cells was recorded and the saline removed. Nitric acid and a drop of caprylic alcohol were then added to the tubes, which were placed in a steam bath until the solution was a clear yellow. The tubes were cooled, the volume of digest recorded, and a 3 ml. aliquot was placed in the cup of a Geiger-Müller counter (Bale, Haven, and LeFevre, 1939) for counting. Afterwards aliquots of the digest were taken for potassium analysis by the method of Shohl and Bennet as modified by Wilde (1939).

Blood for *in vitro* experiments was obtained from rats and rabbits by cutting the throat and in humans by venous puncture. Either heparin or oxalate was used as an anticoagulant. The cells were washed twice in unbuffered mammalian Ringer that was 0.005 molar in potassium (0.037 per cent KCl). For rat and human cells it contained 0.8 per cent NaCl, 0.008 per cent $MgCl_2$, and 0.016 per cent $CaCl_2$, and for rabbit cells 1.0 per cent NaCl, 0.01 per cent $MgCl_2$, and 0.02 per cent $CaCl_2$. The cells were suspended in Ringer containing radioactive potassium or ordinary potassium depending upon the experiment so that 20 cc. of the mixture contained about 1 cc. of cells. The suspension was agitated in a water bath at 37.5°C. and was also aerated by a stream of air. Aliquots were taken at suitable intervals which were measured to the time of starting the centrifuge. The supernatant Ringer was sucked off and counted after a tenfold dilution with water. The cells were washed once with Ringer and treated just as the cells from the *in vivo* experiments. Because of the errors introduced into potassium analyses by large quantities of sodium Ringer was not analyzed for potassium, but the change in the K content was calculated from the change in cell potassium.

In some experiments the change in radioactivity of the Ringer was similarly calculated.

The counts of the standard radioactive potassium solution were plotted on semilogarithmic paper, and activities at times corresponding to sample counts were interpolated from straight lines connecting the plotted points. Ordinarily the semilogarithmic graph had the theoretical slope for a half-life of 12.5 hours. Any deviation from this slope indicated a change in the efficiency of the counter which was thus automatically corrected for by this procedure. All counts were expressed as a fraction of the standard at the time of counting. By dividing this fraction by the ratio of the potassium concentration in the nitric acid digest to that in the standard, solution counts were reduced to a molar basis (*i.e.* number of counts per unit of potassium) which expresses the fraction of the active potassium present in the potassium of the sample.

This figure is referred to as the "activity" of the sample or the relative number of counts per mol of potassium on the basis of 1000 for the number of counts in the standard solution per mol of potassium.

RESULTS

The penetration of radioactive potassium into human cells suspended in Ringer at 37.5°C. is shown in Table I and Fig. 1 as measured in two experiments A and B. The data for cells were fitted empirically by a curve of the type, $x = mt^n + c$, and the slope of this curve at each point calculated as $mnt^{(n-1)}$. The accuracy of each point does not exceed 5 per cent, and this method was considered adequate to give the slope to the possible limits of accuracy. The diffusion coefficient[1] is calculated as the quotient of this slope divided by the difference in relative activity of cells and Ringer. The diffusion coefficients average 0.20×10^{-3} and 0.24×10^{-3} in two cases. The differences are probably not significant.

The penetration of radioactive potassium into the red blood cells of a rabbit *in vivo* is shown in Table II and Fig. 2. The diffusion coefficient has been calculated in the same way as for the human cells, and it is observed to be larger immediately after injection. This might be correlated with the high potassium concentration in the plasma at this time.

Radioactive potassium was put into rabbit erythrocytes by suspending them in Ringer containing active potassium for 10 hours at room temperature (23°C.). A portion of the same cells received identical treatment except that the Ringer contained nonradioactive potassium. Both lots of cells were centrifuged and washed once with plasma. The inactive cells were suspended in radioactive Ringer and the active cells in inactive Ringer. Both lots were aliquoted in 20 ml. portions into 50 ml. Erlen-

[1] This is not strictly a diffusion coefficient in the usual sense, for it has the dimensions of minutes^{-1} and its value depends upon the area and thickness of the diffusing surface as well as the actual speed of penetration.

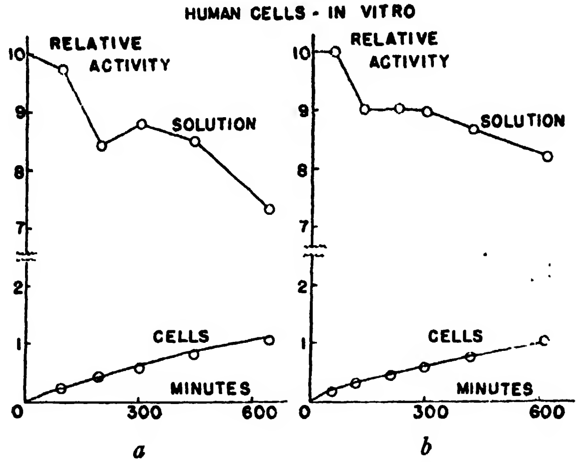

FIG. 1 *a* and 1 *b*. Two experiments on human red cells suspended in Ringer's solution with radioactive potassium (*a*) cells of R.B.D. (*b*) cells of L.F.H. Ordinates, relative activity $\times 10^{-2}$. Abscissae, minutes from time of suspension of the cells in the solution. Curves for cells follow the empirical equations given in Table I.

TABLE I

Human Erythrocytes—in Ringer

A

Time	Hematocrit	K concentration: Ringer	K concentration: Cells	Relative activity: Ringer	Relative activity: Cells	Empirical curve cell activity	Slope cell activity	Diffusion gradient	Diffusion coefficient $\times 10^{-3}$
min.	*per cent*								
0	5.0	5.00	(77.8)	(1000)	0			1000	
95	4.9	5.00	77.8	975	25	24	0.211	950	0.222
195	4.85	5.23	76.6	842	43	43	0.170	799	0.213
300	4.6	5.35	75.5	880	59	65	0.150	821	0.183
445	4.85	5.30	74.4	850	81	86	0.133	769	0.175
640	4.45	5.70	70.4	730	106	110	0.121	624	0.194

Average diffusion coefficient = 0.197×10^{-3} min.$^{-1}$
Empirical curve for cell activity $x = 1.32t^{0.7} - 5.1$

B

Time	Hematocrit	K concentration: Ringer	K concentration: Cells	Relative activity: Ringer	Relative activity: Cells	Empirical curve cell activity	Slope cell activity	Diffusion gradient	Diffusion coefficient $\times 10^{-3}$
0	5.0	5.00	(84.6)	(1000)	0			1000	
60	5.0	5.00	84.6	1000	19	23	0.274	981	0.280
120	4.95	5.07	84.0	900	33	31	0.240	867	0.277
210	4.85	5.13	83.0	900	44	44	0.213	856	0.249
300	5.0	5.13	82.0	896	58	59	0.199	838	0.239
420	4.9	5.30	81.0	863	75	76	0.186	788	0.236
610	5.0	5.55	74.1	817	101	99	0.172	716	0.241

Average diffusion coefficient = 0.254×10^{-3} min.$^{-1}$
Empirical curve for cell activity $x = 0.778t^{0.8} + 3.5$

meyer flasks which were agitated at 37.5°. At suitable intervals pairs of flasks were removed and the cell suspensions treated exactly as in the

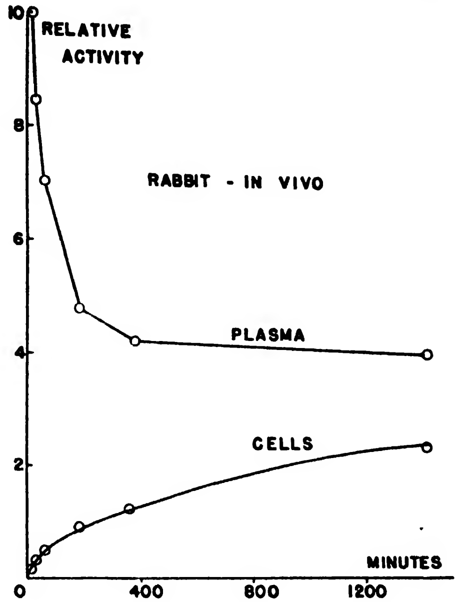

FIG. 2. Relative activity $\times 10^{-2}$ of the potassium (ordinates) in the plasma and blood cells of rabbits drawn at various times (abscissae) after injection of radioactive potassium. Figures in Table II. The curve for the cells is drawn to follow the empirical equation given in Table II.

TABLE II

Rabbit Erythrocytes in Vivo

Time	Hematocrit	K concentration		Relative activity		Empirical curve cell activity	Slope cell activity	Diffusion gradient	Diffusion coefficient $\times 10^{-3}$
		Plasma	Cells	Plasma	Cells				
min.	*per cent*								
10	45.0	6.50	85.6	1000	18	20	1.010	982	0.970
30	40.7	6.15	92.6	846	32	34	0.578	814	0.710
60	40.4	6.34	94.8	705	50	48	0.412	655	0.630
180	37.7	5.60	81.2	480	91	85	0.236	389	0.607
360	34.0	4.94	91.4	422	124	120	0.170	298	0.571
1440	31.6	4.59	93.2	403	236	240	0.084	167	0.503

Average diffusion coefficient = 0.665×10^{-3} min.$^{-1}$
Empirical curve for cell activity $x = 632t^{0.5}$

other experiments. Table III and Fig. 3 show the penetration into and out of the cells. In this case the slopes were estimated graphically. They are less reliable because of the few points which are rather erratic. This may

be due in part to excessive hemolysis. There seems to be a difference between the diffusion constants in the two cases. At present we see no

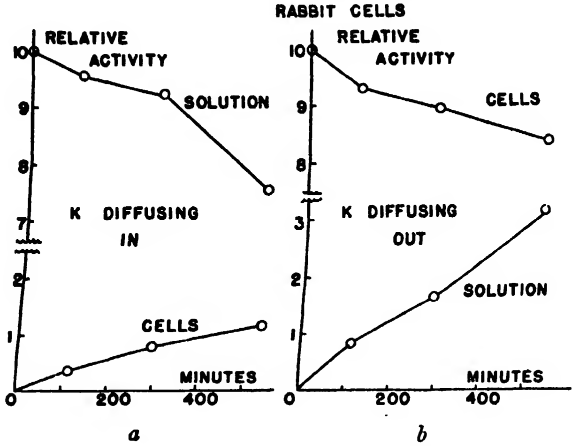

FIG. 3. Rabbit cells *in vitro*. In (*a*) radioactive K is in the Ringer's solution diffusing into the cells; in (*b*) it is in the cells diffusing out into the solution. Ordinates, relative activity of the potassium $\times 10^{-2}$; abscissae time from beginning of diffusion. All curves drawn through experimental points as given in Table III.

TABLE III

Rabbit Erythrocytes in Ringer

A. Diffusion in

Time	Hematocrit	K concentration		Relative activity		Slope cell activity	Diffusion gradient	Diffusion coefficient $\times 10^{-3}$
		Ringer	Cells	Ringer	Cells			
min.	*per cent*							
0	3.0	5.00	80.4	1000	0	0.30	1000	0.30
115	3.1	5.11	73.0	956	38	0.46	918	0.50
295	3.2	5.20	67.7	922	80	0.19	742	0.26
535	2.2	6.42	45.0	756	119	0.15	635	0.23

Average diffusion coefficient = 0.32×10^{-3} min.$^{-1}$

B. Diffusion out

0	3.0	5.00	80.4	0	1000	0.59	1000	0.59
115	3.0	5.11	73.0	84	931	0.36	847	0.42
295	3.2	5.21	67.5	167	895	0.24	728	0.33
535	2.6	5.98	55.3	315	839	0.20	524	0.38

Average diffusion coefficient = 0.40×10^{-3} min.$^{-1}$

justification for considering this difference as significant in view of the inaccuracy of the measurements. The coefficient, however, is probably significantly lower than for the rabbit erythrocytes *in vivo*, but of course

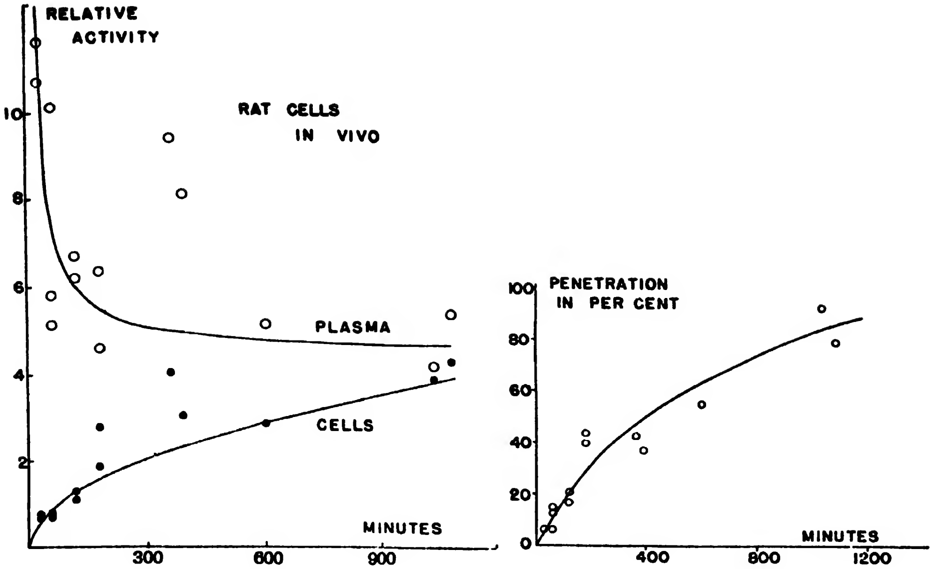

FIG. 4*a*. Results of injections of radioactive potassium into 14 rats. Ordinates, relative activity $\times 10^{-2}$ of the potassium in plasma and cells where the activity of the injected dose in per cent of the body weight is 1000. Data of Table IV.

FIG. 4*b*. Cell activity in per cent of plasma activity or per cent penetration of radioactive K into cells of rats (ordinates) at different times (abscissae) after injection. Calculated from data of Table IV.

TABLE IV

Rat Erythrocytes in Vivo

Time	K concentration		Relative activity		Empirical plasma	Curves cells	Slope cells	Diffusion gradient	Diffusion constant
	Plasma	Cells	Plasma	Cells					
min.									
30	9.1	86	1071	69	1150	64	1.08	1086	1.00
30	8.8	103	1164	76					
60	12.6	84	528	80		92	0.76	653	1.15
60	12.0	95	580	77	750				
60	8.4	96	1030	69					
120	8.2	100	673	113	600	130	0.54	570	0.95
120	8.4	95	622	128					
180	7.6	86	459	182	550	159	0.44	391	1.12
180	9.6		633	278					
360	7.4	78	944	404	500	225			
390	11.0	97	815	304	495	234			
600	11.0	101	514	281	480	290	0.23	290	0.80
1035	6.5	110	421	390	468	382	0.20	86	0.23
1080	7.7	97	544	431	467	390	0.20	57	0.35
						Actual gradient		(113)	(1.77)

Average diffusion coefficient 30 to 600 minutes $= 1.0 \times 10^{-3}$ min.$^{-}$
Empirical curve for plasma $P = 450 + 18{,}000/t$
Empirical curve for cells $x = 11.9t^{0.5}$

the cells *in vitro* were in poor condition. Penetration of radioactive potassium at 23°C. during the loading process took place at a mean rate of 0.1 counts/mol per minute with a gradient of 1000 counts/mol so that the coefficient of diffusion is of the order of 0.1×10^{-3}. This value is 0.27 times the mean rate at 37.5°C. which corresponds to a Q_{10} of about 2.4.

In the course of other experiments a number of rats were injected with radioactive potassium intraperitoneally or subcutaneously. Each animal received 1–3 cc. of 0.1 or 0.2 M KCl, a large dose. The animals were sacrificed after various intervals, blood was collected, and the tissues were analyzed for radioactivity and potassium. The results of these analyses will be reported elsewhere.[2] After centrifuging the blood, plasma was removed as completely as possible and the cells were dissolved in nitric acid without washing. A known volume of plasma was similarly digested in nitric acid, counted, and analyzed for *K*. There is unfortunately a very large variability in the results as shown in Table IV and Fig. 4, probably due to variations in the experimental procedure and to variations in the ability of the different animals to dispose of the large dose of potassium in the various tissues of the body. Nevertheless, if we discard the points at 6 and 6.5 hours, it is possible to make a fair approximation to the data as shown in the curves of Fig. 4 *a*. Then taking the slope of the cell curve by differentiation as before, we have calculated the diffusion coefficient for different points. This value shows very little variation except at 18 hours, and if at this time the actual gradient experimentally observed between cells and plasma is used for calculation instead of the difference between the empirical curves, even this point falls more nearly in line. It is significant, we believe, that the diffusion coefficient is higher in rat than in rabbit or human erythrocytes.

Although these data show great irregularity when plotted as in Fig. 4 *a* they are somewhat more regular if the per cent penetration (*i.e.* cell activity $\times$ 100 $\div$ plasma activity) is plotted against time as in Fig. 4 *b*. In about 30 hours the exchange may be expected to be complete. The interpretation of this curve, however, is somewhat difficult because of the widely varying gradient which was exceedingly high immediately after the injection and because an apparent increase in penetration can be caused by decreased plasma activity due to exchange with other body tissues. The data of Table IV were obtained from experiments on fourteen different rats and they afford therefore most convincing evidence of the cation permeability of rat cells under normal physiological conditions in the body.

Radioactive potassium was loaded into rat cells by injecting active KCl

[2] *Am. Jour. Physiol.*, in press.

into the rat 5 hours before taking the blood. Table V and Fig. 5 show how the active potassium left the red cells when they were suspended in Ringer. There was considerable hemolysis which accounts for the high activity in the Ringer. In this case the points are too erratic to justify fitting an em-

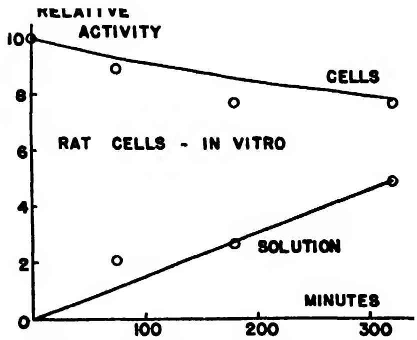

FIG. 5. The diffusion of radioactive potassium from rat red cells into the Ringer's solution in which they are suspended. The graph for the solution follows the empirical formula while the graph for the cells is the calculated theoretical curve (see text). Ordinates relative activity $\times 10^{-2}$; abscissae, time from the beginning of diffusion.

TABLE V

Rat Erythrocytes in Ringer

Time	Hematocrit	K concentration		Relative activity		Empirical curve Ringer activity	Theoretical curve cell activity
		Ringer	Cells	Ringer	Cells		
min.	*per cent*						
0	0.051	5.00	96.5	0	1000	0	1000
75	0.047	5.48	95.0	213	894	115	931
180	0.045	5.83	90.3	264	773	275	860
320	0.032	7.10	89.4	489	769	489	793

Diffusion coefficient $b = 1.0 \times 10^{-3}$

Empirical curve for Ringer activity $R = at = 1.53t$

Theoretical curve for cell activity $x = \left(1000 + \frac{1.53}{0.001}\right)\epsilon^{-.001t} + 1.53t - \frac{1.53}{0.001}$

pirical curve. However, if we assume a linear rise in the Ringer activity $R = 1.53\ t$, and a diffusion coefficient of 10^{-3} the value obtained in the *in vivo* experiment, we can calculate the fall in activity of the cells.[3]

[3] Assuming the law of diffusion

$$\frac{dx}{dt} = b(R - x)$$

and a linear relation for the Ringer activity $R = R_0 + at$ on solving the differential

The upper graph in Fig. 5 is the curve calculated in this way, and it appears to be a reasonable approximation to the experimental points. There seems to be no justification in this data for assuming a different diffusion coefficient for *in vitro* than for *in vivo* exchange in rat erythrocytes.

DISCUSSION

If a cell is permeable to a given ion K and does not change its content of that ion over a period of time, we can say that the numbers of K ions crossing its membrane in each direction in unit time are equal. Now let the fractions x_i and x_o represent the ratios of radioactive to normal K ions inside and out and let p equal the number of K ions crossing in each direction per minute. Then the number of active ions crossing per minute in the two directions is $x_i p$ and $x_o p$.

The change in the number of active ions inside after unit time is $x_o p - x_i p$ or $p(x_o - x_i)$. The rate of change in the ratio of active to total K ions (K_i) is accordingly $\frac{p}{K_i}(x_o - x_i)$. We can let $\frac{p}{K_i} = b$, since K_i must be a constant for a given cell that is not gaining or losing ions, and in differential form we write $dx = b(x_o - x_i)\,dt$. This is identical with the ordinary diffusion equation if we take the relative concentration of radioactive ions to total ions of the kind under consideration. b equals the fraction of ions leaving the cell per minute and is equal to the diffusion coefficient for K ions leaving the cell. The experiments reported here show that the radioactive potassium ions behave as if the membrane were allowing a constant fraction of the potassium inside to cross in each direction per minute. These results are all based on the assumption that the potassium remains in the cells and therefore in so far as they fit diffusion curves they give no evidence of changes in the concentration of potassium due to leakage. Some of the deviations of the diffusion coefficient from a constant value may well represent net movements of potassium. For example, a movement of KCl into the cells increasing their potassium content when the plasma potassium is high would account for the apparently higher rate of uptake of the rabbit cells *in vivo* soon after injection.

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equation we get

$$x = \left(x_0 - R_0 + \frac{a}{b}\right)e^{-bt} + R_0 + at - \frac{a}{b}$$

where x and R are the activities per mol of potassium for the cells and Ringer respectively. x_0 and R_0 are their activities at time 0. t is the time, a the slope of the Ringer activity, and b the coefficient of diffusion.

If the activity of the cell potassium is expressed as a percentage of the activity of the plasma potassium the result also indicates the percentage of the cell potassium which has exchanged with the plasma potassium. Thus in Table I it is seen that after 10 hours *in vitro* 12.4–14.5 per cent of the potassium of human cells has exchanged with the plasma potassium. For rabbit cells the exchange is 29 per cent complete after 6 hours *in vivo* (Table II) and 59 per cent complete after 7.9 hours *in vitro* (Table III). Possibly human cells also would show better exchange *in vivo*. The exchange in rat cells *in vivo* (Table IV) is 55 per cent complete in 10 hours and 79 per cent complete in 18 hours. The comparative constancy of the diffusion constant indicates that in human cells the exchange would be complete if sufficient time were allowed. There is no evidence as yet that the exchange is limited to a certain fraction of the cell K and that this diffusible fraction is larger in rats than in men. It appears instead that the rat cells are more permeable than human cells. The calculated diffusion constants represent a better measure of this permeability than the percentage exchange in unit time because in the latter no account is taken of the varying diffusion gradients.

It should be mentioned that sodium can cause serious contamination in radioactive potassium. K^{41} accounts for less than 7 per cent of the element, and yet it is the only isotope which can be activated to K^{42}. All the sodium atoms can be activated somewhat more easily than the K^{41} isotope. Since radioactive sodium and potassium have very similar half lives, it is easy to see how a small percentage impurity of sodium would cause serious trouble. Metallic potassium which has been used by most other workers as a source of radioactive potassium is notoriously the most difficult form of the element to purify. It may be suggested as a possibility that the low penetration observed by Hahn, Hevesy, and Rebbe (1939 *b*) was due to sodium contamination. The large ratio of plasma to cell sodium would make the counts in plasma very high compared to those in cells and give a very low calculated penetration.

Eisenmann, Ott, Smith, and Winkler (1940)[4] concluded from their measurements with radioactive potassium that there was no free penetration of potassium into human red cells. Actually there is no experimental conflict with our data. Their figures show an average of 4.4 per cent (maximum 8 per cent) penetration in 4 hours whereas our figures show 15 per cent penetration in 10 hours. There are some important differences of tech-

[4] The full report of this work appeared after this manuscript had been accepted for publication. This paragraph was added later by permission of the editors.

nique. Their cells were left in plasma while ours were immersed in Ringer's solution. On the other hand, they added dry potassium to plasma and in such large amounts (up to 60 mEq. per liter of whole blood) that the solution was quite hypertonic. Both of these factors would probably decrease the permeability in their experiments. In our experiments the solution was, if anything, slightly hypotonic which may account for the slight hemolysis observed. Such hemolysis introduced no error into the measurement of the penetration into the cells which remained intact, but the permeability of those cells may have been abnormally high.

The actual penetration of potassium into human cells was not large in our experiments (only 15 per cent). The shape of the curve indicates, however, no great diminution in rate of penetration even after 10 hours. The belief that all the potassium is eventually exchangeable has, therefore, some justification.

It may be supposed that the permeability of the cells may have been modified by the radioactivity of the solutions. According to the results of Mullins (1939) with *Nitella,* the effect if any would be a decrease of permeability. We do not have as yet observations at a sufficient variety of radiation intensities to permit an experimental answer to this question. The actual radioactivity of the solutions in our *in vitro* experiments estimated in terms of the count given by a saturated solution of potassium acetate was about 7 microcuries per liter. This would seem to be low enough so that no isotope effect would be anticipated.

In rats at least there seems to be no escape from the conclusion that the red cells are normally more or less permeable to potassium. Possibly, however, rat cells are impermeable to sodium. Otherwise it would be difficult to understand why potassium does not normally exchange for sodium.

SUMMARY

The diffusion coefficients for the exchange of potassium across the membrane of erythrocytes of humans, rats, and rabbits have been determined by the use of artificially radioactive potassium, both into and out of the erythrocytes both *in vitro* and *in vivo.*

The diffusion coefficients found in minutes^{-1} were 0.2 to 0.25 $\times$ 10^{-3} for human, 0.32 to 0.665 $\times$ 10^{-3} for rabbits, and 1.0 $\times$ 10^{-3} for rat erythrocytes. Rabbit erythrocytes appear to be more permeable *in vivo.*

Reasons are advanced to explain the failure of earlier workers to demonstrate appreciable exchange of potassium in erythrocytes.

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REFERENCES

Bale, W. F., Haven, F. L., and LeFevre, M. L., 1939, *Rev. Scient. Instr.*, **10**, 193.
Cohn, W. E., and Cohn, E. T., 1939, *Proc. Soc. Exp. Biol. and Med.*, **41**, 445.
Eisenmann, A. J., Ott, L., Smith, P. K., and Winkler, A. W., 1940, *J. Biol. Chem.*, **133**, xxviii. 1940, *J. Biol. Chem.*, **135**, 165.
Hahn, L. A., Hevesy, G., and Rebbe, O. H., 1939*a*, *Nature*, **143**, 1021.
Hahn, L. A., Hevesy, G., and Rebbe, O. H., 1939*b*, *Biochem. J.*, London, **33**, 1549.
Joseph, M., Cohn, W. E., and Greenberg, D. M., 1938, *Science*, **87**, 438.
Joseph, M., Cohn, W. E., and Greenberg, D. M., 1939, *J. Biol. Chem.*, **128**, 673.
Manery, J. F., and Bale, W. F., 1940, *Am. J. Physiol.*, in press.
Mullins, L. J., 1939, *J. Cell. and Comp. Physiol.*, **14**, 403.
Noonan, T. R., Fenn, W. O., and Haege, L. F., 1940, *Am. J. Physiol.*, **129**, 432.
Wilde, W. S., *J. Biol. Chem.*, 1939, **128**, 309.

STUDIES ON INVERTEBRATE HEMOGLOBINS (ERYTHROCRUORINS)

By KURT SALOMON

(*From the Department of Physiological Chemistry and Pharmacology, Yale University School of Medicine, New Haven*)

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The most widely distributed respiratory pigments in the animal kingdom are the iron-containing hemoglobins and the copper-containing hemocyanins. The hemocyanins occur only in invertebrates and all have high molecular weights (350,000 to 5,000,000). The hemoglobins, on the other hand, are universally distributed throughout the animal kingdom. Vertebrate hemoglobins, as a rule, have a molecular weight of 68,000 whereas invertebrate hemoglobins (erythrocruorins in Svedberg's nomenclature) vary in their molecular weights from about 34,000 to several millions.

In the present work three erythrocruorins occurring in worms have been studied from a chemical and physical chemical point of view, in order to compare their properties with those of vertebrate hemoglobin. Two very different types of erythrocruorin were studied, *viz.* the macromolecular pigments of the common earth worm (*Lumbricus terrestris*) and of the sand worm (*Nereis virens*) and the low molecular respiratory protein of the so called blood worm (*Glycera dibranchiata* Ehlers).[1] In accordance with the experience of Svedberg (1) the former are freely dissolved in the plasma whereas the latter is locked up in blood corpuscles which are suspended in the body fluid.

Lumbricus erythrocruorin was isolated by repeated salting out or by repeated ultracentrifugation (67,000 × gravity) of purified worm extracts. Beams' air-driven concentrating ultracentrifuge (2) proved to be a suitable tool for the sedimentation and purification of this high molecular pigment. Furthermore the absorption spectra of the three pigments and of some of their derivatives have been studied as well as some of their chemical properties.

The relatively large amount of blood pigment present in *Glycera dibranchiata* Ehlers has made it possible to isolate sufficient quantities of pure

[1] Professor A. Petrunkewitsch of the Osborn Zoological Laboratory was kind enough to identify the species.

crystalline hemin to permit a determination of the configuration of the porphyrin, in order to decide whether the blood heme grouping present in worms is identical with that of vertebrates.

Finally, the dissociation rate of *Lumbricus* and *Glycera* oxyerythrocruorin was compared with that of human oxyhemoglobin in the reaction meter of DuBois (3), in order to determine the rate of dissociation of oxyhemoglobins of large and small molecular size.

EXPERIMENTAL

1. Erythrocruorin of Lumbricus terrestris

Preparation.—200 earth worms (about 500 gm.) were put through a meat mincer and the juice was separated from the fragments by placing the latter on cheese cloth and applying slight pressure. The minced worms were reextracted twice with 0.9 per cent NaCl solution. The brownish red extract thus obtained was centrifuged and the supernatant fluid decanted from the gray precipitate of cell debris. The dark red, turbid solution containing the pigment was mixed with Filter-Cel and filtered through a Buchner filter. A bright red opalescent solution was obtained which was used for most of the qualitative experiments. The preparation of the erythrocruorin extract was carried out in a cold room. This solution is stable for several days, if kept in the refrigerator. Further spinning in the conical head of a laboratory centrifuge at about 3000 R.P.M. failed to produce a precipitate.

From this solution the pigment may be separated by ultracentrifugation or by salting out with ammonium sulfate. The salt was added in small portions to the solution containing the pigment until a grey precipitate was formed. The precipitate was removed by centrifugation and the procedure repeated, until finally the chromoprotein itself was precipitated. The pigment was washed several times with ice cold water.

Chemical Properties.—*Lumbricus* erythrocruorin is soluble in water. After standing about a week in the ice box at pH 7 in phosphate buffer the erythrocruorin solution turns brown and the ferrierythrocruorin band appears at 645 mμ. After the addition of sodium hydrosulfite ($Na_2S_2O_4$) the ferriband disappears. If the solution is then saturated with oxygen, strong oxyerythrocruorin bands reappear and the brown color of the solution turns dark red. All spectroscopic observations were made with a Zeiss pocket spectroscope equipped with a wave length scale. The spectra to be compared were examined simultaneously with the aid of the comparison prism.

The absorption spectra of oxyerythrocruorin (*Lumbricus*) and human oxyhemoglobin appear to be identical. In accordance with some observations of Anson *et al.* (4) we found the ferricompound of *Lumbricus* erythrocruorin different from human ferrihemoglobin insofar as the band in the red is not at 640 mμ, but at 645 mμ (50 Ångstrom units farther towards

the long wave region). At pH 5 it can be shifted by the addition of sodium fluoride to the same position as ferrihemoglobin of man, namely to 610 mμ. As contrasted to vertebrate ferrihemoglobin however, the band is not intensified and sharpened by this procedure, but remains blurred.

The oxidation of a purified solution of oxyerythrocruorin by dyes of various oxidation-reduction potentials was studied. Because many of these dyes absorb light in the same region as ferrihemoglobin, it is difficult or sometimes even impossible to see the ferrihemoglobin band, if formed, with the technique described. In general one may say qualitatively that the pigment is oxidized by the same reagents as hemoglobin, for instance gallocyanin and 2,6 dibromophenol-indophenol produce ferrihemoglobin as well as ferrierythrocruorin in phosphate buffer at pH 7.5. It may be mentioned here that the oxy-bands of *Lumbricus* erythrocruorin persist partially even when an excess of potassium ferricyanide is used. Even dilute solutions of erythrocruorin in water are quite opalescent. Therefore, owing to light scattering, the band of ferrierythrocruorin at 640 mμ is difficult to observe before a large percentage of the chromoprotein is converted to the ferric stage.

In all cases studied, the behavior of a sample of human hemoglobin in phosphate buffer was investigated under identical conditions. The ferribands were identified by shifting them with fluoride to 610 mμ, and by observing their intensification and sharpening by this treatment.

After shaking a solution of oxyerythrocruorin (prepared by ultracentrifugation) at pH 7.5 in phosphate buffer and 25°C. for 24 hours in air, no ferrierythrocruorin could be detected. When a solution of pure oxyerythrocruorin (in phosphate buffer at pH 7.5), prepared by ultracentrifugation, was shaken for 14 hours at 37°C. the respiratory protein was denatured and showed a greenish gray color. That *Lumbricus* erythrocruorin is none the less unable to form a "green hemoglobin" of the kind described by Lemberg and others was shown by the following experiments:

Erythrocruorin was dissolved in secondary phosphate and potassium cyanide was added to the mixture. The system was then aerated. Whereas chicken hemoglobin under identical conditions turned greenish and showed a band at 615 mμ, no green product was formed by *Lumbricus* erythrocruorin, and no band appeared at 615 mμ.

To 5 cc. of a solution of chicken hemoglobin in secondary phosphate 2 mg. of ascorbic acid were added; the pH of the mixture was 7.8. Through this solution, as well as through a correspondingly prepared mixture containing erythrocruorin, air was bubbled for 24 hours at room temperature. No color change took place in the erythrocruorin solution, but the solution

of chicken hemoglobin turned greenish and showed a band at 615–620 mμ. The erythrocruorin solution gave a strongly positive hemochromogen test. No ferrierythrocruorin band was detectable, even after addition of sodium fluoride at pH 5.

A solution of pure oxyerythrocruorin in phosphate buffer at pH 7.5, was treated with CO and the spectrum of carbon monoxide erythrocruorin was compared with that of a solution of human carbon monoxide hemoglobin. The spectra were found to be identical. The readings for the maxima were:

I. 570 mμ II. 535 mμ

Addition of sodium hydrosulfite did not influence the spectrum. It is worth mentioning that the carbon monoxide compound of *Lumbricus* erythrocruorin does not differ appreciably in color from the oxycompound in contrast to the corresponding vertebrate hemoglobin compounds.

The chromoprotein, after being filtered through Filter-Cel, can be adsorbed on aluminum hydroxide at pH 5 and eluted at pH 8. The adsorption of the pigment is only of advantage if purified extracts are used, because a yellow pigment present in crude extracts is adsorbed as well. This pigment remains in the supernatant fluid when the erythrocruorin is sedimented from crude solutions.

Attempts to crystallize *Lumbricus* erythrocruorin by treating solutions of the highly purified pigment (prepared by ultracentrifugation) with ammonium sulfate of various concentrations or with solutions of cadmium sulfate, failed.

2 drops of a concentrated solution of erythrocruorin, prepared by ultracentrifugation, were added to 1 cc. of phosphate buffer of various pH values. Between pH 3.7 and 5.4 precipitates appeared, the strongest one at pH 4.3. No precipitate was formed between 5.4 and 7.3. The isoelectric point determined by electrophoresis, according to Svedberg and Eriksson (5), is at pH 5.28.

2. *Erythrocruorin of Glycera dibranchiata Ehlers*

Preparation.—The main blood vessels of the worm are opened near the head and the blood is collected. Each worm yields about 2 cc. of blood. The blood corpuscles are centrifuged off and washed twice with 0.9 per cent NaCl solution at the centrifuge. Distilled water is added to the washed blood cells to obtain a volume three times that of the original blood volume. A few drops of ether are also added to enhance hemolysis. Repeated freezing and thawing of the corpuscles at −50°C. and +20°C. respectively failed to destroy all of the blood cells present. The addition of the ether caused most of the blood cells to hemolyze, but a certain number remained intact. A similar behavior of the blood corpuscles of *Urechis caupo* was reported by Redfield and Florkin (6). Additional amounts of the erythrocruorin may be obtained as follows. The bodies of the worms are minced, after bleeding, and the mass separated from the juice by submitting it to the same procedure used for the *Lumbricus terrestris* extract. A clear, red purified solution may be obtained by filtration of the extract through Filter-Cel.

Ultracentrifugation.—In this case quantity ultracentrifugation was not practical as a method of purification, since a preliminary run with the analytical centrifuge indicated a molecular weight which is smaller than that of vertebrate hemoglobin. This agrees with the finding of Svedberg (1) that the erythrocruorin of another *Glycera* species, viz., *Glycera goesi*, has a sedimentation constant of 3.5 (m.w. 34,000). A substance with such a molecular weight would require very long sedimentation times with a centrifuge of the type at our disposal.

Electrophoresis.—A solution obtained by laking stored, frozen blood corpuscles was examined in the electrophoresis apparatus of Tiselius with the aid of the Toepler schlieren method. The diagrams obtained by Longsworth's schlieren scanning method (see Fig. 1) showed the presence of two major and of two minor protein components. According to qualitative spectroscopic observations the protein of the lowest mobility represented the *ferri-ferro*erythrocruorin, the protein with the next higher mobility was erythrocruorin. Both minor components had a higher mobility and were colorless. The mobility values obtained at pH 8 with the aid of simple schlieren band photographs were as follows: anode limb: erythrocruorin 1.2×10^{-5}, ferrierythrocruorin 10×10^{-5}; cathode limb: erythrocruorin 0.68×10^{-5}, ferrierythrocruorin 8.3×10^{-5} cm./sec./volt.

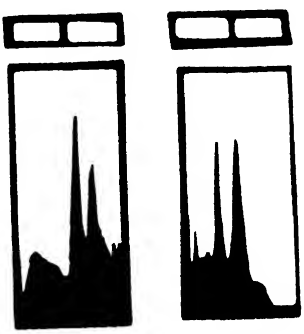

FIG. 1. Electrophoretic diagram of *Glycera* erythrocruorin. Total erythrocruorin concentration approximately 1 per cent; 0.017 molar phosphate pH 8; 180 volts, 3.2 milliamps; 3°C.; Eastman plus X film; Corning filter No. 246; Mazda tungsten filament lamp (300 watt).

Isolation of Hemin.—For the preparation of hemin from *Glycera* erythrocruorin the procedure used by Warburg, Negelein, and Haas (7) for the preparation of *Spirographis* hemin was employed. 240 cc. of a solution of erythrocruorin (two times the original volume of the blood of about 60 worms) were dropped in a mixture of 12 liters of acetone

and 120 cc. normal HCl. The denatured globin was filtered off and the acetone was removed by distilling under diminished pressure, at 35°C., until the crude hemin precipitated. It was dissolved in 30 cc. of hot propionic acid, and 15 cc. of hot HCl (0.5 per cent) were added. The red hemin, which crystallized out, was dried in an evacuated desiccator over sulfuric acid. The yield was 62 mg. of hemin. Blood worm hemin crystallizes in a form identical with that of mammalian hemins.

Preparation of the Mesoporphyrin Dimethyl Ester of Glycera Hemin. 29.6 mg. of hemin were dissolved in a mixture of 1.5 cc. HI (sp.gr. 1.7) and 7 cc. glacial acetic acid, and boiled for 1 minute following the procedure of Fischer and Kögl (8). After cooling to room temperature the mixture was poured into 20 cc. of water containing sodium sulfite and sodium acetate. The porphyrins formed were extracted with ether. The mesoporphyrin fraction was extracted from the ether with 2 per cent HCl. After neutralizing with NaOH the mesoporphyrin was again taken up in ether. The ether solution was dried overnight with sodium sulfate and then evaporated at room temperature under diminished pressure. The mesoporphyrin crystals thus obtained were treated with 15 cc. of freshly prepared, dry methyl alcoholic HCl for 24 hours. The methyl alcohol and HCl were then distilled off under diminished pressure. The violet residue was taken up in a few cc. of chloroform and diluted with ether. From the chloroform-ether mixture the ester was extracted with HCl (5 per cent), and, after neutralizing with NaOH, it was reextracted with ether. The ether was washed several times with water and allowed to stand overnight with sodium sulfate. It was evaporated under nitrogen. A violet, crystalline substance with a melting point of 212°C. (uncorr.) was obtained. The melting point of the ester when mixed with an authentic sample of the synthetic ester prepared in Prof. H. Fischer's laboratory, showed no depression.

Mesoporphyrin dimethyl ester, prepared from *Glycera* hemin: m.p. 212.5°C.
Mesoporphyrin IX, dimethyl ester, synthetic (H. Fischer), m.p. 212°C.
Mixed melting point .... 211.5°C.

The absorption spectra of the two preparations in ether were identical. The absorption bands were found to be at:

I. 490–505 II. 530 III. 570 IV. 630 mμ for the *Glycera* ester;
I. 490–505 II. 530–535 III. 570 IV. 630 mμ for the synthetic ester.

Spectra of Glycera Erythrocruorin and Derivatives. A solution of 0.2 per cent erythrocruorin was compared with a 0.1 per cent solution of human hemoglobin. After addition of potassium ferricyanide both solutions showed the ferrihemoglobin band at 640 mμ, which was shifted to 610 mμ by the addition of sodium fluoride at pH 5 in phosphate buffer. Both solutions showed identical bands at 585 and 545 mμ, after the addition of sodium hydrosulfite. The carbon monoxide compounds of human hemoglobin and *Glycera* erythrocruorin showed identical absorption spectra: I. 570 II. 535 mμ. The ferrierythrocruorin showed a brown color, carbon monoxide erythrocruorin was cherry red; both were similar in color to the corresponding hemoglobin compounds.

Rate of Dissociation of Oxyerythrocruorins

These experiments were kindly performed by Mr. DuBois with the aid of his recording reaction meter (3).

As Roughton and Millikan (9) have shown, the rate of the spontaneous breakdown of oxyhemoglobin into oxygen and hemoglobin may be measured by introducing $Na_2S_2O_4$ into the system. In this instance $Na_2S_2O_4$ does not act as a reducer but it merely absorbs those O_2 molecules which are liberated by the spontaneous dissociation. Consequently the observed rate is independent of the concentration of the hyposulfite (9). In the present experiments the $Na_2S_2O_4$ concentration was 0.5 per cent in borate buffer.

A solution of human hemoglobin of 0.2 per cent Hb was used as the control. The actual concentration of hemoglobin during the reaction was 0.1 per cent. The solutions of worm hemoglobins used were matched by color with the human hemoglobin solution. For the human hemoglobin, when measured at 27°C. and at pH 8.37, the t_{50} value (= half time of reaction) was 0.027 second. The *Lumbricus* erythrocruorin was measured at 23°C. and at pH 8.0; the t_{50} value obtained here was 0.070 second. The solution was opalescent. The photographic record showed only a relatively small deflection. The *Glycera* erythrocruorin was measured at 28°C. and at pH 8.62; the t_{50} value obtained was 0.027 second; *i.e.*, identical with that found for human hemoglobin.

3. Erythrocruorin of Nereis virens

The oxyerythrocruorin of this worm shows absorption bands identical in position with those of human oxyhemoglobin. Addition of potassium ferricyanide produces a ferricompound reacting in the typical fashion with fluoride. The carbon monoxide compound shows a spectrum identical with human carbon monoxide hemoglobin. The pyridine hemochromogen spectra of the two pigments are identical. The *Nereis* pigment was prepared in an analogous way as described for the erythrocruorins of *Lumbricus terrestris* and of *Glycera dibranchiata*, and was also purified by ultracentrifugation. The supernatant shows a dark, brown color. The red pellets obtained by ultracentrifugation dissolved readily in water.

DISCUSSION

The faculty to synthesize iron porphyrin compounds is found in unicellular organisms as well as in highly developed vertebrates. Even in animals which have hemocyanin as their respiratory pigment, hemin is present as an essential part of cytochrome (10). Hemin occurs in certain

respiratory pigments (hemoglobin, erythrocruorin), in myoglobin, in cytochrome *c*, and as the prosthetic group of catalase. The difference in the chemical and physical properties of these chromoproteins depends on the specific protein with which the hemin nucleus is combined, the number of hemin groups present in the molecule, and the character of their linkage to the protein part.

Amphioxus lanceolatus contains no hemoglobin (11) and the Cyclostomata contain hemoglobin of a low molecular weight (1). All other vertebrates, however, contain hemoglobin of the molecular weight of 68,000. Anson *et al.* (4) have pointed out, that significant differences exist in the position of the bands of human hemoglobin and of that of the insect *Chironomus*. These differences are to be attributed to the protein part of the molecule, since the prosthetic group of the *Chironomus* pigment is identical with protoheme IX (12).

The worms are the only class of invertebrates, in which hemoglobin is widely distributed. Two types of hemin have been found in worms: the green-red *Spirographis* hemin and the red protohemin. The constitution of *Spirographis* hemin has been shown by Warburg and his coworkers (13) and by Fischer and von Seemann (14) to be that of 1,3,5,8, tetramethyl-2 formyl,-4 vinyl porphin,6,7, dipropionic acid. It is very similar to hemin IX and is also derived from etioporphyrin III.

The present experiments show that the hemin of *Glycera* is identical with that of the vertebrates. The differences in chemical and physical chemical properties of the respiratory pigment of *Glycera* and that of the vertebrates must therefore be attributed to the protein part of the molecule.

By comparing the half time, t_{50}, of dissociation as measured for human, *Glycera*, and *Lumbricus* oxyhemoglobin with the half time measured for hemocyanins of different molecular sizes, one finds in accordance with Millikan (15), that the order of magnitude of the reaction is the same, even when the molecular size of the pigments is greatly different. It seems that the dissociation time of oxygenated respiratory pigments bears no relation to the structure of the prosthetic group of the molecule and is also independent of the living conditions of the species. Whether this is a general rule cannot be definitely stated before additional measurements on the dissociation rate of other respiratory pigments are available.

SUMMARY

1. Two high molecular invertebrate hemoglobins (the erythrocruorins of *Lumbricus terrestris* and of *Nereis virens*) as well as the low molecular

erythrocruorin of *Glycera dibranchiata* Ehlers were studied. Their physical chemical properties were compared with those of vertebrate hemoglobin.

2. The hemin of the blood pigment of *Glycera dibranchiata* Ehlers was shown to be identical with that of vertebrate hemoglobin.

3. The dissociation rates of *Glycera* and human oxyhemoglobin were measured in the reaction meter of DuBois and t_{50} (half time of the reaction) was found to be identical (0.027 second) for the two pigments. The t_{50} value for the high molecular *Lumbricus* erythrocruorin was 0.070 second.

4. The chemical constitution and physical chemical properties of erythrocruorins were compared with those of vertebrate hemoglobin and of hemocyanin.

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BIBLIOGRAPHY

1. Svedberg, T., *J. Biol. Chem.*, 1933, **103,** 311.
2. Beams, T. W., Linke, F. W., and Sommer, P., *Rev. Scient. Instr.*, 1938, **9,** 248.
3. DuBois, D., *J. Biol. Chem.*, 1940, **137,** 123.
4. Anson, M. L., Barcroft, J., Mirsky, A. E., and Oinuma, S., *Proc. Roy. Soc. London, Series B*, 1924, **97,** 61.
5. Svedberg, T., and Eriksson, Inga-Britta, *J. Am. Chem. Soc.*, 1933, **55,** 2834.
6. Redfield, A. C., and Florkin, M., *Biol. Bull.*, 1931, **61,** 185.
7. Warburg, O., Negelein, E., and Haas, E., *Biochem. Z.*, Berlin, 1930, **227,** 171.
8. Fischer, H., and Kögl, F., *Z. physiol. Chem.*, 1924, **138,** 262.
9. Roughton, F. J. W., and Millikan, G. A., *Proc. Roy. Soc. London, Series A*, 1936, **155,** 258.
10. Ball, E. G., and Meyerhof, B., *J. Biol. Chem.*, 1940, **134,** 438.
11. Redfield, A. C., *Quart. Rev. Biol.*, 1933, **8,** 31.
12. Kirrmann, A., *Bull. Soc. chim. biol.*, 1930, **12,** 1146.
13. Warburg, O., and Negelein, E., *Biochem. Z., Berlin*, 1932, **244,** 9.
14. Fischer, H., and von Seemann, C., *Z. physiol. Chem.*, 1936, **242,** 133.
15. Millikan, G., *J. Physiol.*, 1933, **79,** 158.

STUDIES ON THE LACTASE OF ESCHERICHIA COLI

By H. P. KNOPFMACHER and A. J. SALLE

(From the Department of Bacteriology, University of California, Berkeley)

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A yeast capable of fermenting lactose was first described by Adametz (1889). He found it in his studies on the microorganisms of cheeses and gave it the name *Saccharomyces lactis*. In the same year Beijerinck working with two species of yeast, *Saccharomyces kefir* and *S. tyrocola*, succeeded in demonstrating in the filtrate of his cultures a lactose-hydrolyzing enzyme, which he named "lactase."

Following these investigations lactases were soon detected in many yeasts, molds, bacteria, and in animal tissues. In 1896 Fischer and Niebel voiced the opinion that hydrolysis had always to precede the fermentative decomposition of lactose. From their study of the structure of carbohydrates they concluded that the enzyme concerned must be specific for the alpha-glucose-beta-galactoside linkage of milk sugar. Due to more recent work, however, the validity of these assumptions has become rather questionable.

Lactases are widely distributed in the plant and animal kingdoms. Euler (1922) in reviewing the literature on this subject points out that they are always found in the intestinal tract of young mammals but decrease markedly with age. As to their occurrence in the pancreas there is no agreement among the various authors. More recently Cajori (1935) has reported a lactase from the dog's liver.

Bierry and Ranc (1909) found a lactase in the gastrointestinal tract of the edible snail, *Helix pomatia*, and Wigglesworth (1927) reported it from the midgut of the cockroach, *Periplaneta americana*. It is, however, very doubtful whether these lactases are identical with those of higher animals, and the same holds for the lactases of higher plants, most frequently encountered in the family Rosaceae. The best known example in this group is the enzyme emulsin of bitter almonds, which can hydrolyze lactose as well as beta-glucosides.

Various species of yeasts, molds, and bacteria are capable of fermenting lactose and may contain lactases. Such have been found in *Aspergillus niger* and *A. oryzae* by Hofmann (1934*a*), in *Diplococcus pneumoniae* by Fleming and Neill (1927*a*), in *Clostridium perfringens* by the same authors

(1927*b*), in *Escherichia coli* by Lowenstein, Fleming, and Neill (1929), and in *Escherichia coli mutabile* by Hershey and Bronfenbrenner (1936) and Deere, Dulaney, and Michelson (1936). The presence of lactases in these organisms, however, does not necessarily mean that hydrolysis of the lactose into its constituent sugars has to precede fermentation. The evidence obtained by Willstätter and Oppenheimer (1922) for lactose yeast, by Wright (1936) for *Streptococcus thermophilus*, and more recently by Leibowitz and Hestrin (1939) for maltose yeast points very strongly to the possibility of direct fermentation of lactose and other disaccharides under certain conditions.

Escherichia coli was selected for a general study of its lactase, special emphasis being placed on the kinetics of enzyme action, heat inactivation, and the behavior of the enzyme toward some reducing and oxidizing agents and salts of heavy metals.

EXPERIMENTAL

1. *Preparation of the Enzyme Solution*

Fleming and Neill (1927) were successful in obtaining cell-free extracts of carbohydrases from pneumococci by subjecting them to repeated freezing and thawing. In this process zymases were destroyed, while the activity of the hydrolytic enzymes was preserved. This method is very tedious and time-consuming and therefore was not investigated further.

Hofmann (1934 *b*, *c*) obtained active lactase preparations from *E. coli* and *B. delbrukii* by treating the bacteria with an alcohol-ether mixture and then drying them at room temperature. This method was found to be unsatisfactory in our hands largely because of the susceptibility of the enzyme itself to the solvents used. The activity of preparations of lactase so obtained was very low and decreased on prolonged contact with alcohol, which sometimes was unavoidable.

To obtain appreciable amounts of enzyme, masses of *E. coli* were grown on standard meat extract agar in which 1.5 per cent of lactose had been incorporated. After 48 hours incubation the organisms were washed off with a physiological salt solution, containing 1 per cent of toluene, and subsequently centrifugated. This procedure was repeated three times in order to reduce to a minimum the concentration of adhering metabolic waste products. The resulting suspension contained 6×10^{11} organisms per ml. It was treated with an additional amount of toluene bringing the total concentration of the latter up to 5 per cent. Toluene serves three purposes: (1) It acts as a preservative, (2) it inactivates the zymase complex without affecting the lactase (Willstätter and Oppenheimer, 1922), and (3) it destroys the semipermeability of the cell walls, bringing about a gradual autolysis of the bacteria.

Several attempts were then made to obtain a cell-free enzyme preparation. As was mentioned above it was found that the lactase apparently was very susceptible to alcohol and ether. It was also completely inactivated on dehydration with acetone. When a toluene-treated cell suspension was incubated overnight at 37°C. a dry gelatinous substance was obtained. This was removed and ground to a powder, the relative activity

of which, as determined by a method to be described later, was found to be 82 per cent of that originally present in the bacterial suspension.

The dried powder, consisting of whole cells and cell fragments, was subjected to more rigorous autolysis. Measured portions were suspended in M/15 phosphate buffer solutions of pH 7.0, 8.0, and 9.0 and incubated overnight at temperatures of 37°C. and 46°C. They were then centrifugated and supernatants and sediments tested for lactase activity. The opaque supernatant fluids were practically inactive, whereas the precipitates still exhibited a marked activity though less than that of the dry powder, probably because of the severity of the treatment to which they had been subjected. A microscopical examination revealed that practically all bacterial cells were disintegrated, and only cell fragments were present. The enzyme, apparently, adhered to these cell fragments.

These observations are contrary to reports by Karström (1930), who obtained cell-free lactase preparations from *E. coli* by suspending the dried organisms in phosphate buffer solution of pH 7.0. They are, however, in agreement with results reported by Hershey and Bronfenbrenner (1936), who were unable to separate the enzyme from the bacterial cell and therefore concluded that it was an intracellular water insoluble enzyme.

In another experiment equivalent amounts of toluene-treated cell suspension were exposed to the action of trypsin and papain. In both instances lactase activity was destroyed.

Finally, 120 ml. of bacterial suspension were ground for 18 hours in a ball mill devised by Krueger (1933). But again lactase was inactivated.

In view of these experiences it was decided to use the original cell suspension in all subsequent experiments, and it will be referred to in this report as "enzyme solution" or "*E. coli* lactase" inasmuch as it was solely employed for hydrolyzing lactose. This preparation was stored in an icebox at 5°C. where its activity decreased only slightly during the course of several months.

2. *Materials and Methods*

Standard sugar solutions: 1 gm. of lactose hydrate and glucose, respectively, were dissolved in 100 ml. of distilled water and a few drops of toluene added.

Throughout the course of the experiments dilutions were prepared from these standard solutions, 1 ml. of which contained 10 mg. of the respective sugar.

The Folin-Wu method (1920) for blood sugar determination was chosen as best fitted for measuring the total amount of sugar present before and after hydrolysis by the enzyme.

Experiments were conducted as follows: The desired dilution of the standard was prepared by the use of M/15 phosphate buffers of measured hydrogen ion concentration. One-tenth ml. portions of enzyme preparation were added to 5 ml. of lactose solution and the tubes shaken in a water bath at 36°C. for a certain length of time. Thereupon, they were centrifugated for 30 minutes and the supernatant liquid used for sugar determination. 2 ml. were pipetted into Folin-Wu sugar tubes, 2 ml. of copper solution added, and the tubes then placed in boiling water for 8 minutes. After cooling 2 ml. of color reagent (phosphomolybdic acid) were added, the tubes made up to a volume of 25 ml. with water, and the resulting color compared with that of a standard.

In preliminary readings, employing glucose and lactose solutions of different concentrations, it was found that 1 mg. of lactose corresponded to 0.504 mg. of glucose. In

all experiments, therefore, the values obtained have been expressed in terms of glucose or total reducing sugar on the basis of the above empirical determination.

For example, if the initial concentration of lactose is 1/40 of that of the standard solution, *i.e.* 2 ml. contain 0.5 mg. of lactose, it will be read as 0.252 mg. of glucose or total reducing sugar, a glucose solution being always used as the standard for comparison.

For each experiment a parallel control had to be set up since most of the chemicals whose effect on the enzyme was to be tested were oxidizing or reducing agents, and the enzyme solution itself slightly reduced copper sulfate. For this purpose corresponding amounts of enzyme and chemical reagent were added to 5 ml. of phosphate buffer solution and the reducing values obtained then subtracted from the total.

Finally, a correction for volume had to be made to an extent dependent upon the amount of enzyme solution and chemical reagent added.

It was impossible to maintain a perfectly uniform rate of hydrolysis for the duration of the experiments. The values fluctuate between 51 and 59 per cent hydrolysis per hour for a 1/40 lactose solution. This circumstance, however, was not regarded as of importance inasmuch as the problem selected concerned merely the comparative study of rates of reaction as affected by hydrogen ion concentration, temperature, and chemicals.

RESULTS

1. *The Effect of Hydrogen Ion Concentration*

Optimal conditions with regard to hydrogen ion concentration differ for lactases from various sources (Oppenheimer, 1935).

To determine the effect of pH on the activity of *E. coli* lactase, experiments were carried out as follows: M/15 phosphate buffer solutions of different pH were prepared and their hydrogen ion concentration checked by means of a glass electrode. They were then used to make up lactose solution of a concentration of 1/40 with respect to the standard (0.252 mg. of total sugar per 2 ml.).

As described above, 5 ml. were then mixed with 0.1 ml. of enzyme preparation and shaken in a water bath at 36°C. for 1 hour, and the reducing sugar was determined. The results are given in Table I.

The values are plotted in Fig. 1.

The results indicate that the activity of the enzyme is markedly reduced by slight acidity but much less affected by alkalinity of the medium. The optimum pH for the time period and temperature given seems to extend over the range between 7.0 and 7.5. Consequently, all subsequent experiments were carried out at a pH of 7.5.

2. *The Mechanism of Enzyme Action*

Michaelis and Menten (1913) worked out general rate laws for the action of invertase on sucrose by assuming a chemical combination of the enzyme with its substrate as the governing step in the hydrolysis of the sugar.

The enzyme-substrate equilibrium can be represented by the equation:

$$K_s = \frac{(E)\,(S)}{(ES)}$$

TABLE I

Effect of pH on the Degree of Hydrolysis of Lactose by E. coli Lactase

pH	Amount of total sugar	Hydrolysis	Ratio of activity to that of maximum activity
	mg. per 2 ml.	*per cent*	
5.0	0.254	0.8	0.01
6.0	0.357	41.7	0.71
6.5	0.386	53.2	0.91
7.0	0.399	58.3	0.99
7.5	0.400	58.7	1.00
8.0	0.393	56.0	0.95
9.0	0.381	51.2	0.87

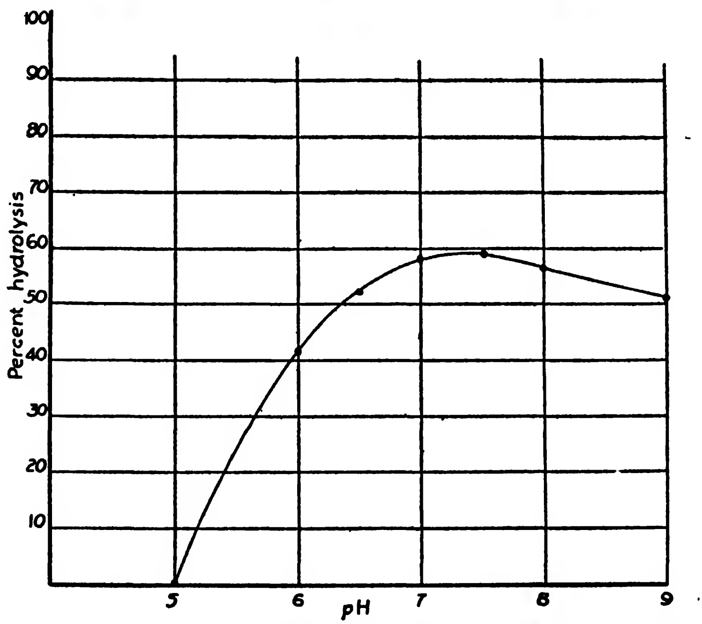

FIG. 1. Effect of pH on the rate of hydrolysis of lactose by *E. coli* lactase.

where (E) and (ES) refer to the concentration of free and combined enzyme respectively and (S) to the concentration of the substrate.

The constant k_s could be determined by simple mathematical calculation, leading to the equation

$$\frac{v}{V_m} = \frac{(S)}{k_s + (S)} \text{ or } k_s = (S)\left(\frac{V_m}{v} - 1\right)$$

in which v represents the initial velocity at the substrate concentration (S), V_m the maximum velocity, k_s, therefore, being equivalent to the substrate concentration at which half the limiting velocity is reached.

Lineweaver and Burk (1934) developed graphic methods for determining dissociation constants of enzyme-substrate compounds. Since in some cases one molecule of enzyme reacts with several molecules of substrate they modified the Michaelis-Menten equation accordingly:

$$k_s = \frac{(E)\,(S)^n}{(ES_n)}$$

and

$$\frac{v}{V_m} = \frac{(S)^n}{(S)^n + k_s}$$

The latter equation can then be written

$$\frac{1}{v} = \frac{k_s}{V_m(S)^n} + \frac{1}{V_m},$$

in which V_m, the maximum velocity, and k_s are constant.

A plot of $\frac{1}{v}$ against $\frac{1}{S^n}$ must therefore give a straight line for some integral value of n. The intercept of this line on the $\frac{1}{v}$ axis is $\frac{1}{V_m}$ and its slope $\frac{k_s}{V_m}$. In this fashion, then, the constants are easily determined.

When the above equation is multiplied by $(S)^n$ it assumes the form $\frac{(S)^n}{v} = \frac{k_s}{V_m} + \frac{(S)^n}{V_m}$. By plotting $\frac{(S)^n}{v}$ against $(S)^n$ a straight line is again obtained. The intercept on the $\frac{(S)^n}{v}$ axis is $\frac{K_s}{V_m}$ and the slope is $\frac{1}{V_m}$.

The latter plot is not only of importance in checking the values obtained by the former but also in discovering any departure from a straight line due to substrate inhibition. In such a case plots of $\frac{(S)^n}{v}$ against $(S)^n$ give curves that rise concavely with increasing substrate concentration.

The following solutions were prepared:

(*a*) A 1/10 dilution of the standard (2 mg. of lactose per 2 ml. = 29.3×10^{-4}M)

(*b*) A 1/20 dilution of the standard (1 mg. of lactose per 2 ml. = 14.6×10^{-4}M)

(*c*) A 1/40 dilution of the standard (0.5 mg. of lactose per 2 ml. = 7.3×10^{-4}M)

(*d*) A 1/60 dilution of the standard (0.33 mg. of lactose per 2 ml. = 4.9×10^{-4}M)

The results of hydrolysis after 30 and 60 minutes are given in Table II.

Upon plotting $1/v$ against $1/S$ and S/v against S practically straight lines were obtained. (See Figs. 2 and 3.) Consequently, it can be concluded that one molecule of enzyme combines with one molecule of lactose as is the case with all the other carbohydrases so far investigated.

TABLE II

Rate of Hydrolysis of Varying Concentrations of the Substrate

	Amount of total sugar	Velocity	$1/v$ (av.)	$1/S$	S/v
	mg. per 2 ml.	*per min.*			
1. Substrate A					
30 min.	1.164	0.0053	192	0.5	384
60 "	1.312	0.0051			
2. Substrate B					
30 min.	0.628	0.0041	260	1.0	260
60 "	0.722	0.0036			
3. Substrate C					
30 min.	0.332	0.0027	392	2.0	
60 "	0.394	0.0024			196
4. Substrate D					
30 min.	0.233	0.0022	500	3.0	167
60 "	0.273	0.0018			

The intercept on the $1/v$ axis is at 138, hence $V_m = 1/138 = 0.0072$ mg. per 2 ml. per minute.

$K_s = V_m \times \text{slope} = 0.0072 \times 132 = 0.95$ mg. per 2 ml. $= 13.9 \times 10^{-4}$M or 0.00139

V_m and k_s, as evaluated from the second plot, are somewhat higher.

The intercept on the S/v axis is at $132 = k_s/V_m$. $1/V_m = 130$, hence $V_m = 0.0077$ and $k_s = 1.02 = 14.9 \times 10^{-4}$M, or 0.00149.

From these plots it may be inferred that the substrate has no inhibiting effect on the rate of hydrolysis by the enzyme.

To determine the effect of different concentrations of *E. coli* lactase on the rate of hydrolysis of lactose an experiment was set up in the ordinary way, using a 1/40 lactose solution but adding varying amounts of enzyme preparation. The results are recorded in Table III.

Plotting the figures of the third column against those of the first gives practically a straight line (Fig. 4). This may be also expressed in a mathe-

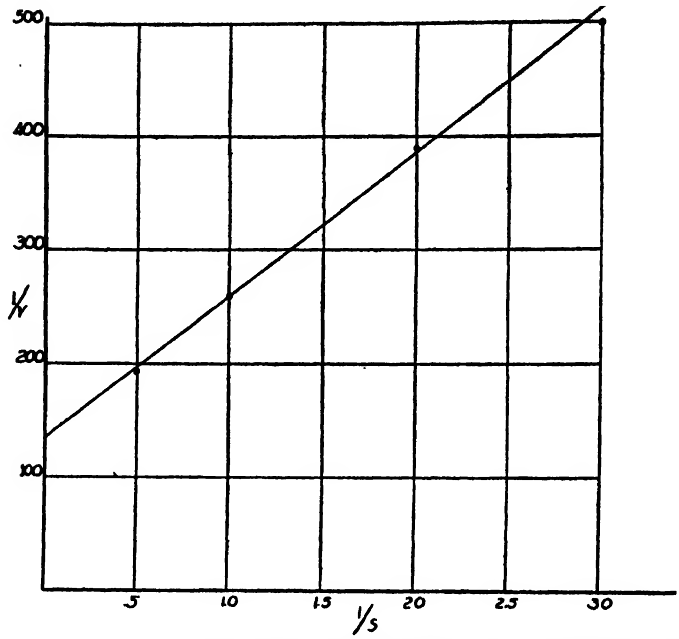

FIG. 2. Nature of the enzyme-substrate intermediate of *E. coli* lactase with lactose.

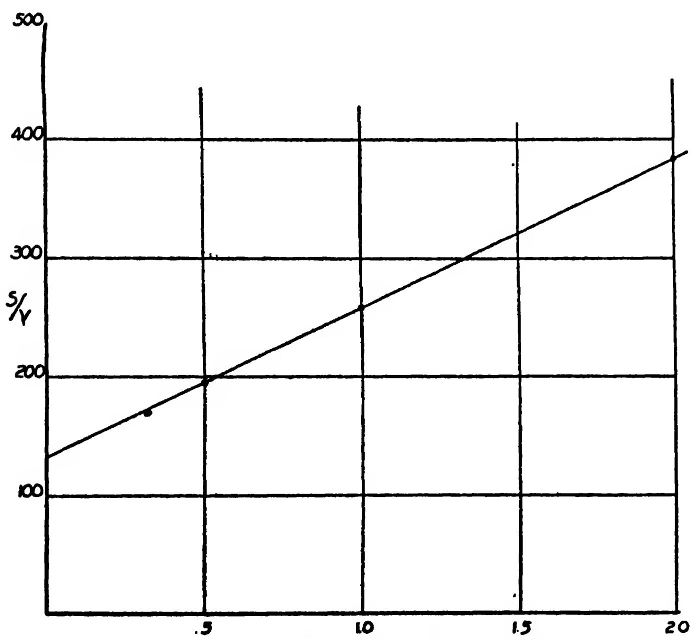

FIG. 3. Test of enzyme-lactose intermediate.

matical form by the equation: $K = \frac{x}{Et}$, where x represents the amount hydrolyzed, E the enzyme concentration, and t the time, which in the above experiment was constant; *viz.*, 1 hour. It is at once evident that

TABLE III

Hydrolysis by Varying Enzyme Concentrations

Amount of enzyme	Amount of total sugar	Amount hydrolyzed (x)	$K = \frac{x}{Et}$	Schütz constant $K_1 = \frac{x}{\sqrt{Et}}$
ml.	*mg. per 2 ml.*			
0.05	0.319	0.067	1.34	0.30
0.10	0.383	0.131	1.31	0.41
0.15	0.452	0.200	1.33	0.52
0.20	0.499	0.247	1.24	0.53

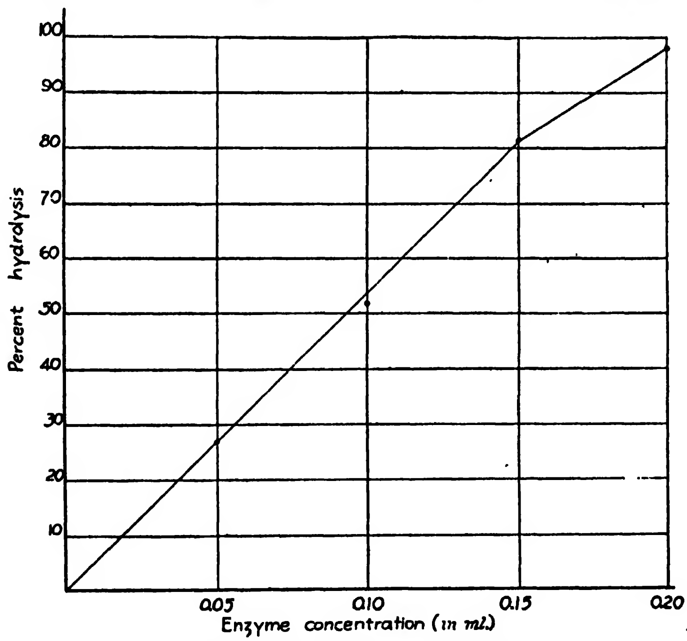

FIG. 4. Relation between enzyme concentration and the rate of hydrolysis.

the values for K fit the above data far better than those for K_1, the so called Schütz constant (1885). Analogous results with yeast lactase were reported by Willstätter and Oppenheimer (1922). In view of the fact that, as has been shown previously, a definite equilibrium between enzyme and substrate is established the products of reaction apparently do not decrease the rate of hydrolysis.

3. *Kinetics of Lactose Hydrolysis by E. coli Lactase*

The hydrolysis of lactose by *E. coli* lactase follows a course between a zero and first order reaction which is quite common for hydrolytic enzymes. Michaelis and Menten (1913), confronted with such difficulty in the

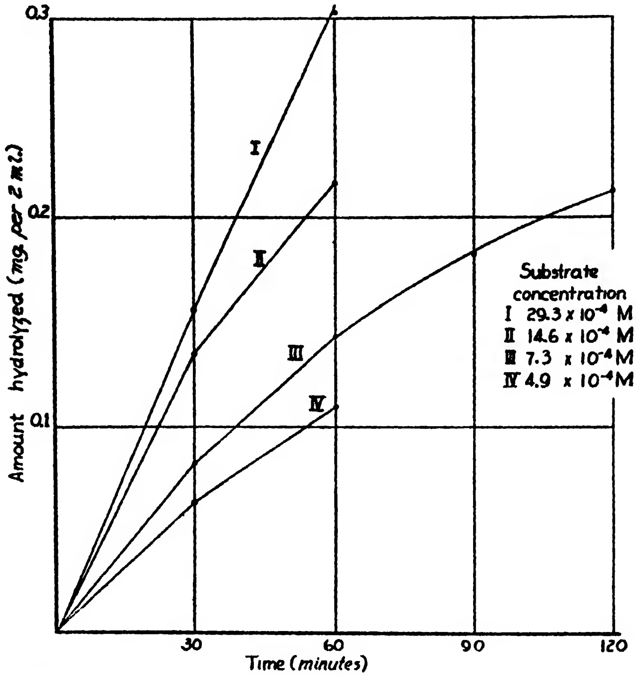

FIG. 5. Relation between substrate concentration and the rate of hydrolysis.

case of invertase, showed that its action could be expressed by a formula that is actually a combination of zero and first order equations.

$$\text{Zero order: } k = k_0 t$$

$$\text{First order: } \ln \frac{a}{a - x} = k_1 t,$$

$$\text{Michaelis-Menten equation: } V_m t = x + k_s \ln \frac{a}{a - x}$$

Essentially the same formula was derived by Van Slyke and Cullen (1914) for the action of urease on urea. Barendrecht (1913) showed that it also held for lactase prepared from yeast.

As suggested in the original paper of Michaelis and Menten, the data of Table II have been presented graphically in two ways. In Fig. 5 the amount hydrolyzed (x) is plotted against time (t). It is readily seen that

for the highest concentration a straight line is obtained indicating that the zero order reaction holds in this case which is in agreement with Michaelis and Menten's observations.

In Fig. 6, $x + 2.3\ k_s \log \frac{a}{a-x}$ is plotted against time, and practically straight lines result, at least for the 1st hour. Only in the case of the 1/40 lactose solution were additional data for the 2nd hour available ($x = 0.185$

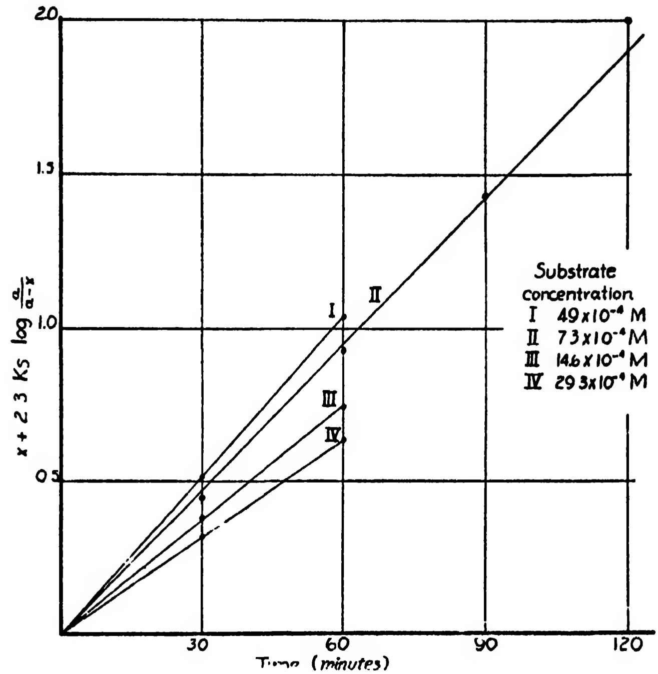

FIG. 6. Kinetics of the hydrolysis of lactose by *E. coli* lactase.

after 1.5 hours and $x = 0.214$ after 2 hours), and these, too, follow practically a straight line. Hence, one can draw the conclusion that hydrolysis of lactose by *E. coli* lactase approximates the reaction course of the integrated Michaelis-Menten equation.

4. *The Effect of Temperature*

5 ml. of a 1/40 lactose solution were incubated with 0.1 ml. of enzyme preparation for 30 minutes at 26°C., 36°C., 46°C., and 56°C. after preheating the enzyme for 5 minutes at the respective temperature. Table IV shows the results obtained.

The recorded drop of lactase activity between 36°C. and 56°C. may be attributed most probably to a more rapid heat inactivation of the enzyme at the higher temperatures.

To elucidate this point further a few experiments were set up designed to determine the rate of enzyme destruction at different temperatures. Test tubes containing measured amounts of enzyme solution were immersed in a water bath at the desired temperature which was closely controlled.

TABLE IV

Effect of Temperature on the Degree of Hydrolysis of Lactose by E. coli Lactase

Temperature	Amount of total sugar	Hydrolysis	Ratio of activity
°C.	*mg. per 2 ml.*	*per cent*	
26	0.295	17.1	
36	0.330	31.0	1.81
46	0.379	50.4	1.63
56	0.249	0	0

TABLE V

Rates of Heat Inactivation of E. coli Lactase at Different Temperatures

Temperature of preheating	Time of preheating	Amount of total sugar	Amount hydrolyzed	First order constant
°C.		*mg. per 2 ml.*		
45	0	0.379	0.127	
	15	0.354	0.102	0.0146
	20	0.345	0.093	0.0156
	30	0.332	0.080	0.0154
53	0	0.379	0.127	
	3	0.336	0.084	0.138
	5	0.319	0.067	0.128
	7	0.300	0.048	0.139

After heating for varying times the tubes were placed in ice water to check as quickly as possible further destruction of enzyme. The residual lactase activity was then determined in the ordinary way of mixing 0.1 ml. of enzyme preparation with 5 ml. of a 1/40 lactose solution and shaking it in a water bath of 36°C. for 1 hour. The results of experiments carried out at temperatures of 45°C. and 53°C. are given in Table V.

It was found that thermal inactivation of *E. coli* lactase followed the equation of a simple first order reaction:

$$2.3 \log A_0/A = kt,$$

where A_0 is the activity of the unheated enzyme solution, in other words, the amount hydrolyzed under ordinary conditions, A the activity of the enzyme heated for the time t, and k the constant of heat inactivation.

The average values for k are thus 0.0152 at 45°C., and 0.135 at 53°C. They can be determined also by plotting log A against t as has been done in Fig. 7.

It is at once evident that the rate constant for heat inactivation changes

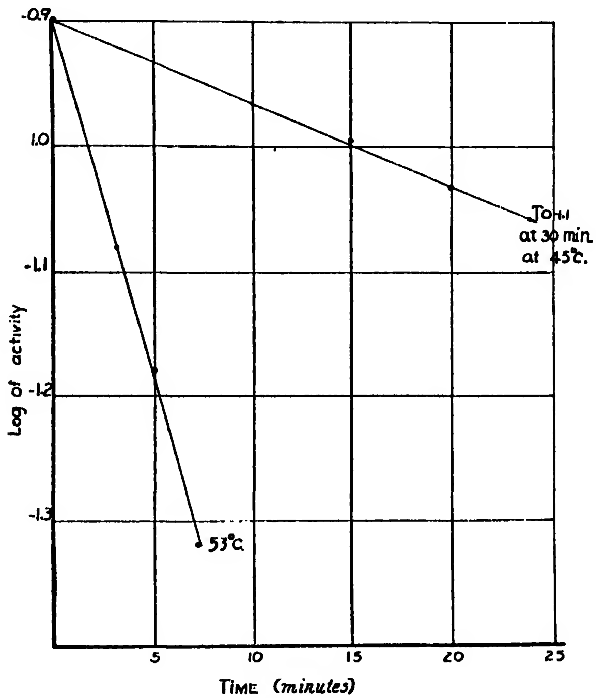

FIG. 7. Rate of heat inactivation of *E. coli* lactase.

very considerably with a relatively small change in temperature. Similar observations have been made with all the enzymes so far investigated, and they are in close agreement with those reported with regard to the denaturation of proteins.

Destruction rates are best considered in their relation to the corresponding "heats of enzyme inactivation." These latter values, known as "critical thermal increments," can be calculated with aid of the van't Hoff-Arrhenius equation

$$\frac{d \ln k}{dt} = \frac{\Delta H}{RT^2}$$

in which k is the reaction velocity constant, T the absolute temperature, R the gas constant, and ΔH the "critical thermal increment."

Integrated between the limits T_2 and T_1, the above equation assumes the following form:

$$\ln \frac{k_2}{k_1} = \frac{\Delta H}{R} \left(\frac{1}{T_1} - \frac{1}{T_2} \right)$$

Since $k_1 = 0.0152$, $k_2 = 0.135$, $T_1 = 318°$, $T_2 = 326°$, and $R = 1.99$ calories, the value of ΔH is calculated as 56,400 calories per mol.

This thermal increment is of the same order of magnitude as those measured in protein denaturation and hence indicates the possibility that *E. coli* lactase may be a protein. Besides, previously cited experiments on destruction of lactase activity through the agency of trypsin and papain constitute another strong evidence for the protein nature of the enzyme.

5. *Activation and Inhibition by Chemicals*

Inhibition phenomena have been most thoroughly investigated with respect to the action of yeast invertase on cane sugar.

Among others, Euler and Svanberg (1920) and Myrbäck (1926) have studied in great detail the inactivation of this enzyme by various chemical reagents. From the results of his experiments Myrbäck has derived some tentative conclusions as to the nature of the groups that enable the enzyme to decompose its substrate. According to him, an acidic, a basic amino, and an aldehydic group are parts of the invertase molecule concerned with the hydrolysis of cane sugar. He found no evidence that sulfhydryl was an essential group.

Only recently, however, Manchester (1939) noted an acceleration of invertase activity due to the addition of potassium cyanide which is in close agreement with the results obtained for *E. coli* lactase. As will be discussed later this may point to the presence of SH groups in the enzyme molecule.

Activation and inhibition of *E. coli* lactase is produced by a variety of chemical agents (see Tables VI–XIII). All experiments were carried out at 36°C. and at a pH of 7.5 for a period of 1 hour. The substrate, as usual, was 5 ml. of a 1/40 lactose solution. One-tenth ml. of enzyme preparation was at first treated with the chemical agent whose action on the rate of hydrolysis was to be determined, by incubation for about 15 minutes and then added to the substrate.

6. *Attempted Reactivation*

Von Euler and Svanberg (1920) succeeded in reactivating, by means of hydrogen sulfide and sodium cyanide, yeast invertase poisoned by heavy metal salts such as silver nitrate and mercuric chloride. A similar reactivation of the protease papain is well known. It was demonstrated first by Vines (1902) and Mendel and Blood (1910) and later by many other investigators.

Only recently, however, were Hellerman, Perkins, and Clark (1933)

TABLE VI

Activation by Potassium Cyanide

Amount of KCN added	Amount of total sugar	Hydrolysis	Ratio of activity to that of untreated enzyme
	mg. per 2 ml.	*per cent*	
None	0.391	55.2	1.00
0.1 ml. 10^{-4}M	0.388	54.0	0.98
0.1 ml. 10^{-3}M	0.418	65.8	1.19
0.1 ml. 10^{-2}M	0.433	72.2	1.31
0.1 ml. 10^{-1}M	0.381	51.2	0.93

TABLE VII

Activation by Sodium Sulfide

Amount of Na_2S added	Amount of total sugar	Hydrolysis	Ratio of activity to that of untreated enzyme
	mg. per 2 ml.	*per cent*	
None	0.380	51.2	1.00
0.1 ml. 10^{-4}M	0.382	51.6	1.01
0.1 ml. 10^{-3}M	0.422	67.4	1.32
0.1 ml. 10^{-2}M	0.412	63.5	1.25

and Hellerman and Perkins (1934) successful in restoring to normal the activity of urease and papain by suitable reducing agents after a preceding inactivation by salts of heavy metals and oxidants.

On the basis of these findings analogous experiments were set up with *E. coli* lactase. They were carried out as usual, the enzyme, however, being treated first with the inhibiting, then with the reducing agent, each for about 15 minutes.

The results are given in Table XIV.

E. coli lactase was irreversibly inactivated by most of the enzymic poisons used, in contrast to yeast invertase, urease, and papain. (See Table XIV.)

TABLE VIII

*Activation by Cysteine**

Amount of cysteine added	Amount of total sugar	Hydrolysis	Ratio of activity to that of untreated enzyme
	mg. per 2 ml.	*per cent*	
None	0.389	54.4	1.00
0.1 ml. 1.8×10^{-3}M	0.388	54.0	0.99
0.1 ml. 4.5×10^{-3}M	0.416	65.1	1.20
0.1 ml. 9.0×10^{-3}M	0.413	64.2	1.18

* Dilutions were prepared from Pfanstiehl's cysteine hydrochloride adjusted to pH 7.0.

TABLE IX

Inhibition by Mercuric Chloride

Amount of $HgCl_2$ added	Amount of total sugar	Hydrolysis	Ratio of activity to that of untreated enzyme
	mg. per 2 ml.	*per cent*	
None	0.395	56.7	1.00
0.1 ml. 10^{-4}M	0.395	56.7	1.00
0.1 ml. 2.0×10^{-4}M	0.353	40.0	0.71
0.1 ml. 3.3×10^{-4}M	0.318	26.0	0.46
0.1 ml. 5.0×10^{-4}M	0.282	11.9	0.21
0.1 ml. 10^{-3}M	0.250	0	0

TABLE X

Inhibition by Silver Nitrate

Amount of $AgNO_3$ added	Amount of total sugar	Hydrolysis	Ratio of activity to that of untreated enzyme
	mg. per 2 ml.	*per cent*	
None	0.398	57.9	1.00
0.1 ml. 10^{-4}M	0.398	57.9	1.00
0.1 ml. 3.3×10^{-4}M	0.311	23.4	0.43
0.1 ml. 5.0×10^{-4}M	0.281	11.5	0.20
0.1 ml. 10^{-3}M	0.252 to 0.260	0 to 3.0	0 to 0.05

TABLE XI

Inhibition by Copper Sulfate

Amount of $CuSO_4$ added	Amount of total sugar	Hydrolysis	Ratio of activity to that of untreated enzyme
	mg. per 2 ml.	*per cent*	
None*	0.382	51.6	1.00
0.1 ml. 10^{-4}M	0.382	51.6	1.00
0.1 ml. 2.0×10^{-4}M	0.351	39.4	0.76
0.1 ml. 3.3×10^{-4}M	0.333	32.1	0.62
0.1 ml. 10^{-3}M	0.262 to 0.270	4.0 to 7.1	0.08 to 0.14

TABLE XII

Inhibition by Iodine (Aqueous Solution in Potassium Iodide)

Amount of I_2 added	Amount of total sugar	Hydrolysis	Ratio of activity to that of untreated enzyme
	mg. per 2 ml.	*per cent*	
None	0.392	55.6	1.00
0.1 ml. 10^{-4}M	0.392	55.6	1.00
0.1 ml. 10^{-3}M	0.324	28.6	0.53
0.1 ml. 10^{-2}M	0.255	1.2	0.02

TABLE XIII

*Inhibition by Hydrogen Peroxide**

Amount of H_2O_2 added	Amount of total sugar	Hydrolysis	Ratio of activity to that of untreated enzyme
	mg. per 2 ml.	*per cent*	
None	0.382	51.6	1.00
0.1 ml. 2.0×10^{-3}M	0.383	52.0	1.01
0.1 ml. 10^{-2}M	0.364	44.3	0.86
0.1 ml. 10^{-1}M	0.361	43.3	0.84

* Dilutions were made from 30 per cent hydrogen peroxide (Merck's Superoxol).

TABLE XIV

Attempted Reactivation of E. coli Lactase

Nature and amount of inhibitor	Nature and amount of reducing agent	Amount of total sugar	Hydrolysis	Ratio of activity to that of untreated enzyme
		mg. per 2 ml.	*per cent*	
None	None	0.382	51.6	1.00
0.1 ml. 10^{-3} M $HgCl_2$	None	0.250	0	0
Same	0.1 ml. 10^{-2} M KCN	0.253	0	0
Same	0.1 ml. 10^{-2} M Na_2S	0.248	0	0
0.1 ml. 10^{-3} M $AgNO_3$	None	0.252 to 0.260	0 to 3.0	0 to 0.06
Same	0.1 ml. 10^{-2} M KCN	0.256	1.6	0.03
Same	0.1 ml. 10^{-2} M Na_2S	0.251	0	0
0.1 ml. 3.3×10^{-4} M $AgNO_3$	None	0.311	23.4	0.43
Same	0.1 ml. 10^{-2} M Na_2S	0.311	23.4	0.43
0.1 ml. 10^{-3} M $CuSO_4$	None	0.262	4.0	0.08
Same	0.1 ml. 10^{-2} M KCN	0.331	31.4	0.61
Same	0.1 ml. 10^{-2} M Na_2S	0.305	21.0	0.41
0.1 ml. 10^{-2} M I_2	None	0.255	1.2	0.02
Same	0.1 ml. 10^{-2} M Na_2S	0.258	2.4	0.05
Same	0.1 ml. 9×10^{-3} M cysteine	0.262	4.0	0.08
0.1 ml. 10^{-3} M I_2	None	0.324	28.6	0.53
Same	0.1 ml. 10^{-2} M Na_2S	0.324	28.6	0.53
0.1 ml. 10^{-2} M H_2O_2	None	0.364	44.3	0.86
Same	0.1 ml. 10^{-2} M Na_2S	0.377	49.9	0.97
Same	0.1 ml. 2×10^{-2} M KCN	0.388	54.0	1.05

Similar observations with heavy metal salts have been reported recently by Winnick, Davis, and Greenberg (1940) with regard to asclepain, a protease from the latex of the milkweed *Asclepias speciosa*. This enzyme is even inhibited by the practically insoluble sulfides of silver and mercury.

In this respect *E. coli* lactase behaves differently. Equal amounts of 10^{-3}M solutions of mercuric chloride and silver nitrate and of a 10^{-2}M solution of sodium sulfide were mixed and then added to the enzyme.

Table XV gives the results obtained.

TABLE XV

Effect of Heavy Metal Sulfides on E. coli Lactase

Nature and amount of chemicals added	Amount of total sugar	Hydrolysis	Ratio of activity to that of untreated enzyme
	mg. per 2 ml.	*per cent*	
None	0.387	53.6	1.00
0.1 ml. 10^{-3} M $AgNO_3$ +0.1 ml. 10^{-2} M Na_2S	0.402	59.5	1.10
0.1 ml. 10^{-3} M $HgCl_2$ +0.1 ml. 10^{-2} M Na_2S	0.386	53.2	0.99

DISCUSSION

In its reaction course *E. coli* lactase obviously follows the general pattern of carbohydrases as best exemplified by yeast invertase.

Studies of heat inactivation and destruction by proteases indicate its protein nature, but beyond this little can be said about the active groups of the enzyme molecule responsible for the decomposition of the substrate.

It is doubtful how far Hellerman, Perkins, and Clark's theory (1933) concerning the oxidation-reduction state of urease may be applicable to *E. coli* lactase. The above investigators postulated sulfhydryl groups to be part of the active enzyme molecule. They contended that oxidation of SH groups to the dithio-stage or the formation of mercaptides with heavy metal ions led to an inhibition of the enzyme studied.

There seems to exist some analogy to their findings in the case of *E. coli* lactase. Its slight activation by reducing agents such as sulfide and cysteine and by cyanide and its readily reversible inactivation by hydrogen peroxide point to easily oxidizible and reducible radicals such as sulfhydryl groups. The same holds for yeast invertase. But if they play any rôle in this connection it is apparently of minor importance. Maybe they are protected in the lactase and invertase molecules or not as actively functional as in proteolytic enzymes. Groups other than sulfhydryl seem to be essen-

tial for enzyme action. Through them the enzyme molecule apparently forms insoluble complexes with mercury and silver ions, whereas cupric ions are bound in a looser combination.

As for the action of iodine one might speculate on the formation of addition compounds such as have been suggested by Herriott (1936) in the case of pepsin. He was able to isolate diiodo-tyrosine from pepsin that had been inactivated previously by treatment with iodine. On the other hand, he noticed no appreciable oxidation of the enzyme by iodine.

SUMMARY

A "lactase solution" was prepared from *Escherichia coli*. The mechanism of its action has been studied and changes in the rate of hydrolysis under various conditions investigated.

The hydrolysis of lactose by the enzyme approximates the course of reaction of the integrated Michaelis-Menten equation. One molecule of enzyme combines with one molecule of substrate.

E. coli lactase is readily inactivated at pH 5.0, and its optimal activity at 36°C. is reached between pH 7.0 and pH 7.5.

The optimal temperature for its action was found to be 46°C. when determinations were carried out after an incubation period of 30 minutes.

Its inactivation by heat follows the course of a first order reaction, and the critical thermal increment between the temperatures of 45°C. and 53°C. was calculated to be 56,400 calories per mol.

The enzyme is activated by potassium cyanide, sodium sulfide, and cysteine, and irreversibly inactivated by mercuric chloride, silver nitrate, and iodine.

After inactivation with copper sulfate partial reactivation is possible, while the slight inhibition brought about by hydrogen peroxide is completely reversible.

The possible structure of the active groups of *E. coli* lactase as compared with other enzymes has been discussed.

BIBLIOGRAPHY

Adametz, L., Saccharomyces lactis, eine neue Milchzucker vergährende Hefeart, *Centr. Bakt.*, 1889, **5**, 116.

Barendrecht, H. P., Enzyme action, facts and theory, *Biochem. J.*, London, 1913, **7**, 549.

Beijerinck, M. W., Die Lactase, ein neues Enzym, *Centr. Bakt.*, 1889, **6**, 44.

Bierry, H., and Ranc, A., Dédoublement du lactose et de ses dérivés par les lactases animales, *Compt. rend. Soc. biol.*, 1909, **66**, 522.

Cajori, F. A., The lactase activity of the intestinal mucosa of the dog and some characteristics of intestinal lactase, *J. Biol. Chem.*, 1935, **109**, 159.

Deere, C. J., Dulaney, A. D., and Michelson, T. D., The utilization of lactose by *Escherichia coli-mutabile*, *J. Bact.*, 1936, **31**, 625.

Euler, H. v., Chemie der Enzyme, Munich and Wiesbaden, J. F. Bergmann, 1922, **2**, 284.

Euler, H. v., and Svanberg, O., Über Giftwirkungen bei Enzymreaktionen, *Fermentforschung*, 1920, **3**, 330.

Fischer, E., and Niebel, W., Über das Verhalten der Polysaccharide gegen einige thierische Secrete und Organe, *Sitzungsber. Akad. Wissensch. Berlin*, 1896, 73.

Fleming, W. L., and Neill, J. M., Studies on bacterial enzymes. III. Pneumococcus maltase and lactase, *J. Exp. Med.*, 1927*a*, **45**, 169.

Fleming, W. L., and Neill, J. M., Studies on bacterial enzymes. V. The carbohydrases and lipases of the Welch bacillus, *J. Exp. Med.*, 1927*b*, **45**, 947.

Folin, O., and Wu, H., A system of blood analysis. A simplified and improved method for determination of sugar, *J. Biol. Chem.*, 1920, **41**, 367.

Hellerman, L., Perkins, M. E., and Clark, W. M., Urease activity as influenced by oxidation and reduction, *Proc. Nat. Acad. Sc.*, 1933, **19**, 855.

Hellerman, L., and Perkins, M. E., Activation of enzymes. II. Papain activity as influenced by oxidation-reduction and by the action of metal compounds, *J. Biol. Chem.*, 1934, **107**, 241.

Herriott, R. M., Inactivation of pepsin by iodine and the isolation of diiodo-tyrosine from iodinated pepsin, *J. Gen. Physiol.*, 1936, **20**, 335.

Hershey, A. D., and Bronfenbrenner, J., Dissociation and lactase activity in slow lactose-fermenting bacteria of intestinal origin, *J. Bact.*, 1936, **31**, 453.

Hofmann, E., Untersuchungen über Glykoside und Disaccharide spaltende Enzyme von Schimmelpilzen, *Biochem. Z.*, Berlin, 1934 *a*, **273**, 198.

Hofmann, E., Neues zur Frage nach der Spezifität der Glykosidasen, insbesondere bei Schimmelpilzen und Bakterien, *Naturwissenschaften*, 1934 *b*, **22**, 406.

Hofmann, E , Über das Vorkommen von Glukosidasen beziehungsweise Galaktosidasen und Disaccharide spaltenden Enzymen in Bakterien, *Biochem. Z.*, Berlin, 1934 *c*, **272**, 133.

Karström, H., Zur Spezifität der α-Glucosidasen, *Biochem. Z.*, Berlin, 1930, **231**, 399.

Krueger, A. P., New type of ball mill for maceration of tissues and bacteria under aseptic conditions, *J. Infect. Dis.*, 1933, **53**, 185.

Leibowitz, J., and Hestrin, S., The direct fermentation of maltose by yeast, *Enzymologia*, 1939, **6**, 15.

Lineweaver, H., and Burk, D., The determination of enzyme dissociation constants, *J. Am. Chem. Soc.*, 1934, **56**, 658.

Lowenstein, L., Fleming, W. L., and Neill, J. M., Studies on bacterial enzymes. VII. Lactase and lipase of the colon bacillus, *J. Exp. Med.*, 1929, **49**, 475.

Manchester, T. C., Note on the acceleration and retardation of invertase activity, *J. Biol. Chem.*, 1939, **130**, 439.

Mendel, L. B., and Blood, A. F., Some peculiarities of the proteolytic activity of papain, *J. Biol. Chem.*, 1910, **8**, 177.

Michaelis, L., and Menten, M. L., Die Kinetik der Invertinwirkung, *Biochem. Z.*, Berlin, 1913, **49**, 333.

Myrbäck, K., Über Verbindungen einiger Enzyme mit inaktivierenden Stoffen, *Z. phys. Chem.*, 1926, **158**, 160.

Oppenheimer, C., Die Fermente und ihre Wirkungen, 1935, suppl. 1, 265.

Schütz, E., Eine Methode zur Bestimmung der relativen Pepsinmenge, *Z. phys. Chem.*, 1885, **9**, 577.

Van Slyke, D. D., and Cullen, G. E., The mode of action of urease and of enzymes in general, *J. Biol. Chem.*, 1914, **19**, 141.

Vines, S. H., Tryptophane in proteolysis, *Ann. Bot.*, 1902, **16**, 1.

Wigglesworth, V. B., Digestion in the cockroach. II. The digestion of carbohydrates, *Biochem. J.*, London, 1927, **21**, 797.

Willstätter, R., and Oppenheimer, G., Über Lactasegehalt und Gärvermögen von Milchzuckerhefen, *Z. phys. Chem.*, 1922, **118**, 168.

Winnick, T., Davis, A. R., and Greenberg, D. M., Physicochemical properties of the proteolytic enzyme from the latex of the milkweed, *Asclepias speciosa* Torr. Some comparisons with other proteases. I. Chemical properties, activation-inhibition, pH-activity, and temperature-activity curves, *J. Gen. Physiol.*, 1940, **23**, 275.

Wright, H. D., Direct fermentation of disaccharides and variation in sugar utilization by *Streptococcus thermophilus*, *J. Path. and Bact.*, 1936, **43**, 487.

THE SULFHYDRYL GROUPS OF EGG ALBUMIN*

By M. L. ANSON

(*From the Laboratories of The Rockefeller Institute for Medical Research, Princeton, New Jersey*)

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INTRODUCTION

The first part of this paper describes a number of ways of estimating the SH groups of denatured egg albumin by measuring how much of a particular SH reagent is needed to abolish the SH groups and by measuring how much ferricyanide is reduced by the SH groups. The same titration value is obtained whether the SH groups are abolished by the oxidizing agents, ferricyanide and tetrathionate, or the heavy metal compound, *p*-chloromercuribenzoate; whether the titration is carried out in a guanidine hydrochloride solution or in a solution of Duponol PC, a detergent consisting of long chain alkyl sulfates; whether the abolition of the nitroprusside test or the reduction of ferricyanide is used as proof of the abolition of the SH groups. The same amount of ferricyanide is reduced by denatured egg albumin whether the reduction is carried out in a solution of guanidine hydrochloride, urea, or of Duponol PC. This agreement between the SH values obtained by very different procedures is strong evidence of the validity of the results.

Ferricyanide is a particularly convenient titrating agent. It is readily available and stable. Under the conditions used ferricyanide reacts almost immediately with the SH groups of denatured egg albumin and yet does not react with other protein groups. Altogether, denaturation by guanidine hydrochloride or Duponol PC and oxidation of the SH groups by ferricyanide can be carried out in a few minutes.

It is important to use reagents of suitable purity for the titrations in guanidine hydrochloride solution. I have found that some samples of guanidine hydrochloride and of protein contain impurities which bring about the abolition of SH groups and thus interfere with the nitroprusside test and the SH titrations in guanidine hydrochloride solution. A method has accordingly been worked out for obtaining pure guanidine hydro-

* A brief account of the SH titration methods has already been published (Anson, 1940 *b*).

chloride, and the techniques of the nitroprusside test and the SH titrations in guanidine hydrochloride solution have been so modified as to minimize interference by impurities.

Whether or not the SH groups of native egg albumin react with a particular SH reagent depends on which SH reagent is used. All the SH groups of egg albumin can be abolished by reaction of the native form of the protein with iodine and some can be abolished by reaction of the native form of the protein with iodoacetamide (Anson, 1940 *a*) despite the fact that the SH groups of native egg albumin do not give a pink color with nitroprusside (Heffter, 1907; Arnold, 1911), and are not oxidized by cystine (Mirsky and Anson, 1935), ferricyanide (Mirsky and Anson, 1936), or porphyrindin (Kuhn and Desnuelle, 1938). The present experiments show further that *p*-chloromercuribenzoate, which combines firmly with the SH groups of denatured egg albumin, combines with native egg albumin either not at all or at least much more loosely than it combines with denatured egg albumin.

The reactions of iodine and native egg albumin (Anson, 1940 *a*) have now been studied in more detail. It has been found that if a small amount of iodine is added in the cold, the SH groups of neutral native egg albumin are all abolished without oxidation of many of the SH groups beyond the S-S stage and without conversion of many tyrosine groups into di-iodotyrosine groups. If enough iodine is added, the SH groups are oxidized beyond the S-S stage, the S-S groups originally present are oxidized, and the tyrosine groups are converted into di-iodotyrosine groups.

The present pictures of protein structure are not complete enough to provide detailed explanations of the various reactions of the SH groups of different native and denatured proteins with different SH reagents. The facts about the properties of protein groups such as SH groups, however, are important for the development of an adequate theory of protein structure.

The SH titrations in guanidine hydrochloride solution which were worked out with egg albumin can be applied to tobacco mosaic virus. Furthermore, the SH groups of the virus, like the SH groups of egg albumin, can be abolished by reaction of the native form of the protein with iodine. No reaction other than the iodine reaction is known by which the SH groups of native egg albumin and tobacco mosaic virus can be abolished. The discovery of the iodine reaction has thus made possible the study of the biological properties of tobacco mosaic virus which has been modified by oxidation of its SH groups by iodine. The chemical and biological experiments with tobacco mosaic virus, which were suggested by the experiments with egg albumin, will be described in other papers.

Previous Estimations of the SH Groups of Denatured Egg Albumin.—The various procedures which have been used to estimate protein SH groups (Mirsky and Anson, 1935; Kuhn and Desnuelle, 1938; Greenstein, 1938; Anson, 1939) are all similar in principle to the methods used to estimate the SH groups of simple SH compounds such as cysteine and glutathione. SH reagents in general react less readily with protein SH groups than with the SH groups of cysteine. Some SH reagents, furthermore, can, under suitable conditions, react with protein groups other than SH groups. The problem, therefore, in the estimation of the SH groups of unhydrolyzed protein is to find conditions under which the SH reagent reacts with all the protein SH groups and no other groups. These conditions have apparently been fulfilled in the estimation of the SH groups of egg albumin by two methods which were developed from the earlier work. In the first method, one measures how much porphyrindin has to be added to denatured egg albumin in guanidine hydrochloride solution so that all the protein SH groups are oxidized and the protein no longer gives a pink color with nitroprusside (Greenstein, 1938). In the second method, one measures how much ferricyanide is reduced by denatured egg albumin in a solution of the detergent, Duponol PC (Anson, 1939).

Ferricyanide and porphyrindin are added to denatured rather than to the native egg albumin because native egg albumin does not reduce ferricyanide and porphyrindin at all. Guanidine hydrochloride or Duponol PC are added because in the absence of such substances not all the SH groups even of denatured egg albumin are rapidly oxidized by dilute ferricyanide and porphyrindin. The nitroprusside test is carried out in guanidine hydrochloride solution but not in Duponol PC solution because the SH groups of denatured egg albumin give a strong pink color with nitroprusside in guanidine hydrochloride solution but only a negligible pink color in Duponol PC solution. The ferricyanide reduction can be carried out in guanidine hydrochloride and urea solution as well as in Duponol PC solution but the estimation of the ferrocyanide formed as Prussian blue is less convenient than when the reaction is carried out in Duponol PC solution.

Various tests were carried out by Greenstein and myself to show that ferricyanide and porphyrindin under the conditions used react specifically and completely with the SH groups of denatured egg albumin. Since no one of these tests is conclusive, the validity of the SH estimations has been tested by comparing the results obtained by different procedures.

SH Titrations in Guanidine Hydrochloride Solutions.—In the porphyrindin titration as originally carried out (Greenstein, 1938) guanidine hydrochloride is added to a neutral protein solution, the solution is allowed to stand

45 minutes, porphyrindin is added to the protein denatured by the guanidine hydrochloride, and finally nitroprusside and ammonia are added to see whether enough porphyrindin has been added to oxidize all the SH groups.

The new titrations in guanidine hydrochloride solution are carried out as follows. To 0.5 cc. of 2 per cent native egg albumin or tobacco mosaic virus there are added 0.1 cc. of neutral phosphate buffer, 0.5 cc. of ferricyanide, tetrathionate or *p*-chloromercuribenzoate[1] solution, and 1.2 gm. of guanidine hydrochloride of tested purity. 3 minutes later test is made for the abolition of the SH groups either by seeing whether the protein gives a nitroprusside test in the presence of dilute cyanide or by seeing whether the protein can still reduce ferricyanide in Duponol PC solution. The concentration of titrating agent is found which just suffices to abolish the SH groups.

The new titrations differ from the porphyrindin titration in that different titrating agents are used; interference by impurities is minimized by using especially purified guanidine hydrochloride, adding the titrating agent before the guanidine hydrochloride, and carrying out the nitroprusside test in the presence of cyanide; the ferricyanide reduction test as well as the nitroprusside test is used to prove the abolition of SH groups; and the whole titration is carried out in the presence of phosphate buffer. I shall now discuss the reasons for the changes which have been made.

In the new titrations ferricyanide, tetrathionate, and *p*-chloromercuribenzoate are used as titrating agents instead of porphyrindin. The substitution of the ferricyanide and tetrathionate for porphyrindin makes the titration in guanidine hydrochloride solution safer and much more convenient. Porphyrindin is hard to prepare, unstable, and a dangerously strong oxidizing agent. Although porphyrindin reacts first with the SH groups of denatured egg albumin in guanidine hydrochloride solution it also in time reacts with other groups. Ferricyanide and tetrathionate are readily available and are weaker oxidizing agents than porphyrindin.

The inclusion of mercuribenzoate as a titrating agent provides a good test for the SH specificity of the titration. Whereas porphyrindin, ferricyanide, and tetrathionate oxidize SH to S-S, mercuribenzoate combines with but does not oxidize SH groups. It is conceivable that the oxidizing agents might oxidize protein groups other than SH groups or that mercuribenzoate might combine with groups other than the SH groups. The SH group, however, is the only protein group known to react with both oxidizing agents and heavy metal compounds.

Formaldehyde abolishes the SH groups of denatured egg albumin in guanidine hydrochloride solution only if the formaldehyde is added in great excess. Formaldehyde cannot, therefore, be used as a titrating agent.

The following observations show that some samples of guanidine hydrochloride contain impurities which bring about the abolition of SH groups and so interfere with the

[1] *p*-chloromercuribenzoate has been used as an SH reagent by Hellerman (1937; 1939).

nitroprusside test and that this interference can be diminished by cyanide. 5 mg. of denatured egg albumin gives a strong pink color with nitroprusside in guanidine hydrochloride solution. I have found, however, that the pink color obtained is much stronger with some samples of commercial guanidine hydrochloride (Eastman or Hoffman-La Roche) than with others. When a guanidine hydrochloride is used which gives a weak color, then the color is weaker the more guanidine hydrochloride is used and the longer the denatured protein is allowed to stand in guanidine hydrochloride solution before the addition of nitroprusside. A sample of guanidine hydrochloride which gives a weak color gives a strong color if it is first recrystallized. If 1 drop of 0.1 N cyanide is added to the protein solution before the addition of guanidine hydrochloride, then a strong nitroprusside test is obtained with all samples of guanidine hydrochloride. Even when a strong nitroprusside test is obtained without cyanide, cyanide slows up the rate of fading of the pink color. On the other hand, the rate of fading can be enormously increased by adding an amount of copper sulfate equivalent to only 10 per cent of the SH groups present. The results which have been summarized do not definitely prove how the impurities in guanidine hydrochloride bring about the abolition of SH groups. They suggest, however, that the impurities are in part, at least, heavy metal compounds which catalyze the oxidation of the SH groups of denatured egg albumin by oxygen and that cyanide inhibits this oxidation of protein SH groups by oxygen by combining with the heavy metal impurities. It is known that heavy metal compounds can catalyze the oxidation of the SH of cysteine (references in Bernheim and Bernheim, 1939) and of denatured egg albumin (Rosenthal and Voegtlin, 1933) by oxygen.

The cyanide added to diminish the effects of impurities in the nitroprusside test is too small in amount to cause any appreciable reduction of S-S to SH. Cystine and denatured egg albumin whose SH groups have been oxidized to S-S groups do not give any color with nitroprusside in guanidine hydrochloride solution even when 1 drop of 0.1 N cyanide is added. The ease with which the S-S groups of a denatured protein are reduced by cyanide varies from protein to protein. 1 drop of 0.1 N cyanide is safe for those proteins which I have tried, but it may not be safe for all proteins.

The following experiments show that impurities in guanidine hydrochloride reduce the amount of the ferricyanide needed to abolish the SH groups of denatured egg albumin, and that the effect of impurities is much less in the new titration procedure in which the titrating agent is added before the protein is denatured by guanidine hydrochloride than in the old titration procedure in which the protein is allowed to stand in guanidine hydrochloride solution before the addition of the titrating agent. When satisfactory guanidine hydrochloride is used the amount of ferricyanide needed to abolish the nitroprusside test is the same whether the ferricyanide is added before the addition of guanidine hydrochloride or 30 minutes after the addition of guanidine hydrochloride. With one poor sample of Eastman guanidine hydrochloride (not the worst), nine-tenths the normal amount of ferricyanide was required to abolish the nitroprusside test if the ferricyanide was added before the addition of guanidine hydrochloride, but only five-tenths the normal amount if the ferricyanide was added 30 minutes later.

The discrepancy between the amount of ferricyanide needed to abolish the SH groups of egg albumin when the ferricyanide is added before the guanidine hydrochloride and when it is added 30 minutes after the guanidine hydrochloride can be used as a test for the purity of the guanidine hydrochloride. When such a test is applied to commercial guanidine hydrochloride, usually, in my experience, the product is found to be unsatis-

factory. Guanidine hydrochloride cannot be purified by recrystallization without great loss. I have therefore found conditions for the effective and economical purification of guanidine carbonate. Guanidine hydrochloride prepared from purified guanidine carbonate is satisfactory for SH titrations.

Greenstein (1938) found that the SH groups of his egg albumin were stable in neutral guanidine hydrochloride solution. His sample of guanidine hydrochloride was therefore satisfactory.

Although one can always make sure that one has pure guanidine hydrochloride and pure egg albumin, not all proteins can readily be obtained in as pure a state as egg albumin. With some proteins it is particularly important to have a titration procedure like the present one which minimizes the effects of impurities and to carry out tests for the presence of impurities. The origin of the present detailed experiments with guanidine hydrochloride, in fact, was my inability to obtain a constant value for the SH content of tobacco mosaic virus when different samples of guanidine hydrochloride and virus were used.

The same titration value is obtained if the nitroprusside test on the albumin treated with the titrating agent is carried out almost immediately after the addition of the titrating agent and the guanidine hydrochloride or 30 minutes after the addition of the titrating agent and guanidine hydrochloride. The waiting has therefore been eliminated and the time needed for the titration very much shortened.

Instead of using the disappearance of the nitroprusside test as an indication that all the SH groups have been abolished one can use the failure to reduce ferricyanide. After the titrating agent and guanidine hydrochloride have been added to the protein, the protein is precipitated and washed with trichloracetic acid, the precipitate is dissolved in neutral Duponol PC solution, ferricyanide is added, and a test is made for ferrocyanide. If either the nitroprusside test or the ferricyanide test for SH were insensitive or not specific for SH then different titration values would be obtained by using these two very different SH tests for the end point. Since the nitroprusside test is more convenient than the ferricyanide reduction test if the titration is carried out in guanidine hydrochloride solution, the ferricyanide reduction test is used not as a routine procedure but only as a check on the validity of the titrations.

The whole SH titration is in all cases carried out in a neutral solution buffered with phosphate. Phosphate establishes a reproducible pH. In the absence of phosphate, furthermore, a pink color is formed immediately on the addition of nitroprusside to a neutral or even slightly acid guanidine hydrochloride solution of denatured protein and the color becomes stronger on the subsequent addition of ammonia. Phosphate conveniently prevents the formation and fading of the pink color before the addition of ammonia.

Cyanide must not be present during the titrations with ferricyanide or mercuribenzoate despite the fact that it is desirable to have cyanide present during the nitroprusside test. For cyanide combines with mercuribenzoate and inhibits the oxidation of protein SH groups by ferricyanide. The mechanism of this inhibition has not been studied. It is possible that the oxidation of protein SH groups by ferricyanide is catalyzed by heavy metal impurities which combine with cyanide. Dilute cyanide does not prevent

the oxidation of the SH groups of denatured egg albumin in neutral guanidine hydrochloride solution by tetrathionate. (Since submitting this paper I have found that cyanide inhibits almost completely the oxidation of SH groups by ferricyanide, tetrathionate, and the uric acid reagent provided the concentration of cyanide is high enough and that copper and zinc ions promote these oxidations.)

In agreement with the results previously obtained by the porphyrindin titration method the equivalent of 1 cc. of 0.001 M ferricyanide, tetrathionate, or mercuribenzoate is needed to abolish the SH groups of 10 mg. denatured egg albumin in guanidine hydrochloride solution whether the abolition of the nitroprusside test or the failure to reduce ferricyanide is used as an end point. It should be emphasized that the agreement between the new titrations and the porphyrindin titration in its original form exists only when the samples of protein and guanidine hydrochloride used happen to be free of impurities which interfere with the original titration method much more than they do with the new methods.

Rate and Completeness of SH Reactions in Neutral Guanidine Hydrochloride Solution.—As we have seen, if 1 cc. of 0.001 M ferricyanide, tetrathionate, or mercuribenzoate is added to 10 mg. of denatured egg albumin in neutral solution and ammonia and nitroprusside are added 3 minutes later, no pink color is obtained. The question arises, does the SH reagent react with the protein SH groups in the neutral solution, or after the addition of ammonia, or is the colored compound of the nitroprusside test formed but destroyed by the SH reagent before it can be observed? Ferricyanide and mercuribenzoate can rapidly destroy the color formed in the nitroprusside reaction. The experiments designed to answer this question show that the abolition of the SH groups in neutral solution by ferricyanide and mercuribenzoate is completed in 3 minutes but that part of the tetrathionate reaction takes place after the addition of ammonia.

The following experiments show that the oxidation of the SH groups by ferricyanide takes place in the neutral solution. After the addition of the ferricyanide to the neutral protein solution the solution is colorless, indication of reduction of the brown ferricyanide to the colorless ferrocyanide. If the protein which has been treated with ferricyanide is precipitated and washed with trichloracetic acid, a protein precipitate is obtained which is free of ferricyanide and has been exposed to ferricyanide in neutral but not in alkaline solution. The SH groups of this protein have been abolished. The protein gives no nitroprusside test when dissolved in guanidine hydrochloride solution and does not reduce ferricyanide in neutral Duponol PC solution.

Further experiments show that tetrathionate, like ferricyanide, can oxidize all the SH groups of denatured egg albumin in neutral solution but that the oxidation by tetrathionate is slower than the oxidation by ferricyanide and so is not complete in 3 minutes.

The trichloracetic acid precipitate of the albumin treated for 3 minutes with tetrathionate in neutral guanidine hydrochloride solution still gives a moderately strong nitroprusside test in guanidine hydrochloride solution and reduces about half as much ferricyanide in Duponol solution as untreated albumin. Since no nitroprusside test is obtained if ammonia and nitroprusside are added directly after 3 minutes, part of the tetrathionate reaction responsible for the abolition of the nitroprusside test must take place after the addition of ammonia. If the tetrathionate is allowed to stand 30 minutes in the neutral guanidine hydrochloride solution before the addition of trichloracetic acid, then it is found that the SH groups have all been abolished by the reaction in neutral solution.

Finally, the evidence that mercuribenzoate combines with the SH groups of denatured egg albumin in neutral guanidine hydrochloride solution. If first 1 cc. of 0.001 M mercuribenzoate and then 1 cc. of 0.001 M ferricyanide are added to 10 mg. of denatured egg albumin in neutral guanidine solution, the brown color of the ferricyanide persists. If the mercuribenzoate had not combined with and protected the SH groups, the ferricyanide would have been reduced to colorless ferrocyanide. When mercuribenzoate combines with SH groups of urease it similarly protects them from oxidation by porphyrindin (Hellerman, 1939).

The compound between mercuribenzoate and the SH groups of denatured egg albumin in neutral guanidine hydrochloride solution is dissociated by trichloracetic acid. If denatured egg albumin which has combined with mercuribenzoate is precipitated with trichloracetic acid and dissolved again with guanidine hydrochloride it gives about as strong a nitroprusside test as egg albumin which has never been exposed to mercuribenzoate.

Measurement of Ferricyanide Reduction in Guanidine Hydrochloride and Urea Solution. –1 cc. of 0.001 M ferrocyanide is formed when ferricyanide is reduced by 10 mg. of denatured egg albumin in Duponol PC solution (Anson, 1940 *a*) or, as in the present experiments, in guanidine hydrochloride or urea solution. The amount of ferrocyanide formed is within wide limits independent of the ferricyanide concentration.

The estimation of SH groups by ferricyanide reduction is more convenient in Duponol PC than in guanidine hydrochloride or urea solution. Duponol PC, unlike guanidine hydrochloride and urea, prevents the precipitation of denatured egg albumin by the acid ferric sulfate added for the estimation of ferrocyanide as Prussian blue. Duponol PC interferes with the development of Prussian blue less than guanidine hydrochloride and in neutral solution denatures egg albumin more rapidly than urea.

Denaturation in neutral urea solution is slow and egg albumin loses some of its SH groups on standing in neutral urea solution if ordinary commercial urea is used and the urea solution does not contain cyanide. In the present experiments, therefore, denaturation by urea is brought about in acid solution in which denaturation is rapid and SH groups are more stable. When the acid urea solution containing 10 mg. of denatured egg albumin is neutralized, 1 cc. of 0.001 M ferricyanide is added. 1 cc. of 0.001 M ferrocyanide is formed and the protein when precipitated with trichloracetic acid and redissolved with guanidine hydrochloride gives no nitroprusside test. The nitroprusside test of untreated egg albumin in guanidine hydrochloride solution is much more intense than the test in urea solution.

I have not been able to confirm the conclusion of Greenstein (1938) that urea "liberates" fewer SH groups from egg albumin than guanidine hydrochloride. Even if 1 cc. of 0.001 M ferricyanide and urea are added to a neutral solution of 10 mg. of egg albumin which has not been treated with acid, the protein after being precipitated by trichloracetic acid no longer gives a pink color with nitroprusside in guanidine hydrochloride solution.

As will be described elsewhere, the SH groups of egg albumin can be estimated by the blue color given with the uric acid reagent, the SH value being the same as that obtained by the present methods. When unhydrolyzed albumin is used the reaction is carried out in urea solution. When albumin partially hydrolyzed by pepsin or acid is used, the presence of urea is not necessary.

Urea promotes the oxidation not only of the SH groups of denatured egg albumin but also the oxidation of free cysteine, tyrosine, and tryptophane. Partial hydrolysis "activates" not only SH groups but the few other protein groups I have tried.

SH Titrations in Duponol PC Solution.—In neutral Duponol PC solution as in neutral guanidine hydrochloride solution 1 cc. of 0.001 M ferricyanide, tetrathionate, or mercuribenzoate is required to abolish the SH groups of 10 mg. of denatured egg albumin.

After the SH groups of denatured egg albumin have been abolished by ferricyanide or tetrathionate, the protein when precipitated by trichloracetic acid and redissolved in guanidine hydrochloride solution no longer gives a nitroprusside test. As in guanidine hydrochloride solution, the ferricyanide reaction is more rapid than the tetrathionate reaction and cyanide interferes with the ferricyanide reaction but not with the tetrathionate reaction.

After mercuribenzoate has combined with all the SH groups of denatured egg albumin in neutral Duponol PC solution the protein no longer reduces dilute ferricyanide. Thus when the SH estimation is carried out in Duponol PC solution as when it is carried out in guanidine hydrochloride solution, it is possible to titrate the SH groups with both an oxidizing agent and a heavy metal compound and to use both the nitroprusside reaction and the ferricyanide reduction as tests for the abolition of the SH groups.

The ferricyanide reduction test should not be used after the tetrathionate reaction because the decomposition products formed from tetrathionate in acid solution reduce ferricyanide. The nitroprusside test should not be used after the mercuribenzoate reaction because trichloracetic acid dissociates the compound between mercuribenzoate and SH.

The Reactions of Iodine and Native Egg Albumin.—Despite the fact that native egg albumin does not react with nitroprusside, ferricyanide, or porphyrindin, all the SH groups of egg albumin can be abolished by reaction of the native form of the protein with iodine. Native egg albumin which has reacted with iodine no longer gives a nitroprusside test when denatured

nor does it reduce ferricyanide in Duponol PC solution (Anson, 1940 *a*). I have now studied the reactions between iodine and neutral native egg albumin in somewhat more detail, mainly in order to compare the egg albumin reactions with the reactions between neutral native tobacco mosaic virus and iodine, which will be described elsewhere.

By adding iodine in acid solution it is possible to oxidize the SH groups of native egg albumin without converting the tyrosine groups into di-iodotyrosine groups. Iodine abolishes the SH groups of native egg albumin even at pH 3.2 (Anson, 1940 *a*). At pH 3.2 iodine does not react with free tyrosine, or with the proteins pepsin (Herriott, 1937), and chymotrypsinogen (Anson, 1940) which contain tyrosine but not cysteine. The present experiments show that it is also possible in neutral solution to oxidize the SH groups of native egg albumin without converting many tyrosine groups to di-iodotyrosine groups or oxidizing many of the SH groups beyond the S-S stage.

If 1.3 cc. of 0.001 N iodine is added to 10 mg. of native egg albumin at 0°C., all the iodine is absorbed as shown by a negative starch test. All the SH groups are abolished as shown by a negative nitroprusside test in guanidine hydrochloride solution. Theoretically it takes 1 cc. of 0.001 N iodine to oxidize the SH of 10 mg. of egg albumin to S-S. The excess 0.3 cc. of 0.001 N iodine actually added is not sufficient to cause much further oxidation of the sulfur groups to RSOH, RSO_2H, or RSO_3H, or to convert many tyrosine groups into di-iodotyrosine groups. The 10 mg. of egg albumin treated with 1.3 cc. of 0.001 N iodine still gives a strong nitroprusside test in guanidine hydrochloride solution, if the protein is exposed to strong cyanide in alkaline guanidine hydrochloride solution before the addition of nitroprusside, indicating S-S groups which are reduced to SH by alkaline cyanide. The 10 mg. of egg albumin which has absorbed 1.3 cc. of 0.001 N iodine still gives a strong purple color when boiled with Millon's reagent. Free di-iodotyrosine (Vaubel, 1900) and, as Harrington and Neuberger (1936) have shown, insulin whose tyrosine groups have been iodinated do not give a color with Millon's reagent.

Since submitting this paper I have found conditions under which the absorption of only 1 cc. of 0.001 N iodine by 10 mg. of native egg albumin brings about the abolition of the nitroprusside test in guanidine hydrochloride solution.

If in the reaction between native egg albumin and iodine, the concentration of iodine and the time and temperature of the reaction are high enough, then all the SH and S-S groups are oxidized beyond the S-S stage, as shown by a negative cyanide-nitroprusside test, and all the tyrosine groups are converted into di-iodotyrosine groups, as shown by a negative Millon test. The conditions for abolishing the cyanide-nitroprusside test are roughly the same as those for abolishing the Millon test.

I have not done any experiments to find out whether the iodine added to

native egg albumin reacts with any groups other than the SH and tyrosine groups.

Egg albumin whose SH groups have been abolished by iodine does not abolish the SH groups of untreated egg albumin in neutral guanidine hydrochloride solution.

Reactions of p-Chloromercuribenzoate with Cysteine and Native Egg Albumin.—In this section it will be shown that mercuribenzoate combines with native egg albumin either not at all or at least much more loosely than it combines with cysteine or with the cysteine in denatured egg albumin.

I have found that the compound between cysteine and mercuribenzoate, like the compound between cysteine and aldehyde (Schubert, 1936) and the cysteine in native egg albumin,[2] does not give a nitroprusside test or reduce ferricyanide but does reduce iodine. Thus the nitroprusside and ferricyanide tests cannot be used to find out whether mercuribenzoate has combined with the SH groups of native egg albumin because these groups do not react with nitroprusside and ferricyanide even when they are not combined with mercuribenzoate. On the other hand, the iodine reaction cannot be used either because SH reduces iodine even when it is combined with mercuribenzoate. I have accordingly used an indirect procedure involving the addition of free cysteine. If mercuribenzoate added to egg albumin is tightly bound to the protein, it cannot combine with added cysteine and the added cysteine is then free to reduce ferricyanide.

If first 1 cc. of 0.001 M cysteine and then 1 cc. of 0.001 M ferricyanide are added to 10 mg. of either native egg albumin or to denatured egg albumin in Duponol PC solution, the ferricyanide is reduced by the cysteine. In the absence of cysteine, native egg albumin does not reduce ferricyanide under any conditions and denatured egg albumin does not reduce ferricyanide under the conditions used, namely, low temperature, dilute ferricyanide, and short time of reaction.

If 1 cc. of 0.001 M mercuribenzoate is added to the *native* albumin before the addition of cysteine and ferricyanide, the ferricyanide is not reduced. This shows that the cysteine has combined with the mercuribenzoate. Either the mercuribenzoate does not combine with the native protein or it is rapidly withdrawn from its combination by the addition of cysteine. In contrast, if the mercuribenzoate is added to *denatured* egg albumin before the addition of cysteine and ferricyanide, the ferricyanide is reduced. Mercuribenzoate remains attached to the SH groups of denatured egg albumin, for a short time at least, even if cysteine is added.

SH Groups and Protein Structure.—The present results and indeed all the work on the SH groups of egg albumin and other proteins show that a

[2] I do not mean to suggest that the SH in native egg albumin is linked to aldehyde or heavy metal or in any other way. It seems to me more likely on the basis of the present inconclusive evidence that the SH groups of native egg albumin are not linked.

reagent which reacts with the SH groups of free cysteine may or may not react with cysteine bound in a protein. Whether or not the reaction takes place depends on what SH reagents and proteins are used, on the concentration of these substances and the time, temperature, and pH of the reaction, on whether the protein is native or denatured, on whether the solution of denatured protein contains substances such as guanidine hydrochloride or Duponol PC, and on whether the solution contains catalysts such as zinc and copper salts or inhibitors such as cyanide.

It would, of course, be desirable to be able to explain the now rather extensive experimental results in terms of some theory of protein structure. The facts, however, although they lead to vague general conclusions about the structural changes involved in denaturation, do not as yet provide proof of any definite, detailed picture of the structural relationships of SH groups in native and denatured proteins. *A priori*, a protein SH group may fail to react with an SH reagent because the protein SH group is inaccessible, or bound, or made unreactive by neighboring protein structure. *A priori*, several of these factors may operate at once, or one factor may be decisive under one set of conditions, another factor decisive under a different set of conditions. As more facts accumulate the arbitrary assumptions which can be made in connection with the three kinds of structural theories become more and more restricted.

It should be remembered that in some proteins S-S (Walker, 1925) and tyrosine (Mirsky and Anson, 1936) groups which are not detectable in the native protein are detectable by the same tests in the denatured form of the protein. The problem of how denaturation and other changes in protein structure produce changes in the properties of protein groups is not peculiar to SH groups.

It should also be remembered that the SH groups of cysteine are more readily oxidized in neutral than in acid solution and that the SH groups of cysteine are more readily oxidized than the SH groups of glutathione (Anson, 1939). Thus molecular structure can greatly influence the properties of SH groups even in relatively simple SH compounds.

EXPERIMENTAL

Reagents.—Egg albumin is thrice recrystallized with ammonium sulfate, dialyzed, and stored frozen.

Duponol PC (Du Pont) is stored at room temperature as a filtered 10 per cent stock solution.

A 5 per cent solution of ground sodium nitroprusside is made fresh daily and stored in ice water. Nitroprusside dissolves slowly unless it is first ground.

The phosphate buffer consists of equal parts of 1.0 M Na_2HPO_4 and NaH_2PO_4.

For the ferricyanide titration reagent grade potassium ferricyanide is used. When the amount of ferrocyanide formed from ferricyanide is measured either a correction is made for the ferrocyanide present in commercial ferricyanide or the ferrocyanide is removed by oxidation with bromine (Anson, 1939). Bromine in addition to oxidizing ferrocyanide to ferricyanide also brings about some other reactions which result in darkening of the solution. This darkening is greater in the original procedure in which the bromine is added step-wise than in the following simpler procedure in which the bromine is added in dilute solution all at once, and in excess. 0.6 M ferricyanide is made up and centrifuged to remove insoluble matter. The ferrocyanide impurity is estimated by adding to 2 cc. ferricyanide solution 7 cc. of water, 0.5 cc. of 2 N sulfuric acid, and 0.5 cc. of ferric sulfate solution. The amount of red light absorbed by the Prussian blue formed is compared with the red light absorbed by a known amount of ferrocyanide in the absence of ferricyanide. Saturated bromine water is diluted 75 times with water and assumed to be 0.005 N which allows for a 10 per cent loss on dilution. If it is desired to know the concentration of bromine in the dilute solution accurately, an excess of iodide is added to a sample and the iodine liberated by bromine is titrated with thiosulfate. Twice the amount of bromine theoretically needed to oxidize the ferrocyanide present is added to the ferricyanide solution and water is added to make the ferricyanide 0.4 M. After the solution has stood 20 minutes the excess bromine is removed by aeration and the solution is allowed to stand overnight before being used. The purified ferricyanide is stored in the cold in a dark bottle. Since in the course of months ferrocyanide forms in the ferricyanide solution the ferricyanide solution is occasionally tested for ferrocyanide with ferric sulfate.

Ferric sulfate containing gum ghatti is prepared according to Folin and Malmros (1929).

A stock 0.1 M thiosulfate solution containing 0.1 gm. sodium carbonate per liter is standardized with the iodine formed by the reaction between iodide and iodate (Peters and Van Slyke, 1932).

The stock 0.1 iodine solution contains 0.18 N KI. The stock 0.1 N iodine previously used (Anson, 1940*a*) contained only 0.12 N KI and lost some iodine on dilution with water. The iodine solution is first made up roughly and then titrated with thiosulfate.

Tetrathionate is formed by adding just enough thiosulfate to iodine to abolish the starch test. It is made up just before being used.

p-chloromercuribenzoic acid is prepared according to the directions of Whitmore and Woodward (1932) and dissolved as the sodium salt. The solution is stable for a few days at least if stored frozen. I am indebted to Dr. Leslie Hellerman for the mercuribenzoic acid.

The guanidine hydrochloride used in the present experiments was a satisfactory batch of the Eastman product. Since guanidine hydrochloride is very hygroscopic it is first dried in a desiccator, then distributed in a number of small containers which are tightly stoppered, and stored in the cold or in some dry atmosphere.

Most samples of the commercial guanidine hydrochloride have not proved satisfactory. Good guanidine hydrochloride should dissolve in an equal weight of water to give a clear and colorless solution. The color given with egg albumin and nitroprusside in guanidine hydrochloride solution should be the same as when guanidine hydrochloride recrystallized from water is used and should not be increased if 1 drop of 0.1 N cyanide is present. The rate of fading of the color should not be much greater than that obtained

with recrystallized guanidine hydrochloride. Letting the albumin stand in neutral guanidine hydrochloride solution for 30 minutes before the addition of nitroprusside should not decrease the color obtained when nitroprusside is added. The same amount of ferricyanide should be required to abolish the nitroprusside test of denatured egg albumin in guanidine hydrochloride solution whether the ferricyanide is added before or 30 minutes after the addition of guanidine hydrochloride. Details of the nitroprusside test and the ferricyanide titration are given in later sections.

Guanidine hydrochloride can be recrystallized from water with a yield of 21 per cent. This recrystallization is used to obtain a good product for comparative purposes, not to prepare the bulk of guanidine hydrochloride used. 20 gm. of guanidine hydrochloride are dissolved in 16 cc. of water at 50°C. The solution is cooled in salt ice water and the guanidine hydrochloride is filtered off in the cold on a pre-cooled Buchner funnel. After the filter cake has been sucked and pressed as dry as possible it is placed in a desiccator.

As much as 5 gm. of guanidine hydrochloride can be dissolved in 1 cc. of water at 100°C. On cooling, however, too thick a suspension of solid is obtained for purification purposes.

Recrystallization from 80 per cent alcohol gives a 55 per cent yield and always improves the product. A completely satisfactory product is obtained from a single recrystallization, however, only if the original amount of impurities is not too great. 20 gm. of guanidine hydrochloride are dissolved in 11.4 cc. of 80 per cent alcohol at 100°C. The solution is brought about as quickly as possible, since guanidine hydrochloride gradually changes into a water-insoluble material at high temperatures. The solution is cooled and filtered as before, washed with cold absolute alcohol, and dried in a vacuum desiccator over NaOH.

Satisfactory guanidine hydrochloride can be prepared from purified guanidine carbonate. *Guanidine carbonate is not hygroscopic and it is much less soluble than the hydrochloride.*

First, guanidine carbonate (American Cyanamid) is stirred up with twice its weight of water and filtered. If the first part of the filtrate is not perfectly clear it is refiltered. An equal volume of 95 per cent alcohol is added to the filtrate with mechanical stirring. The resulting suspension is cooled to 0°C., filtered in the cold on a Buchner funnel, washed with cold 95 per cent alcohol, and sucked and pressed as dry as possible. Concentrated hydrochloric acid is added to the solid carbonate first with hand stirring and when the suspension becomes fluid with mechanical stirring. When the fizzing on the addition of a drop of acid becomes weak, 1.0 N hydrochloric acid is added to complete the neutralization to green to brom thymol blue. 1 or 2 cc. of water are added to a drop of guanidine hydrochloride solution before the indicator test. The solution should remain green to the indicator even after continued stirring since the carbon dioxide formed is not removed immediately. The solution is allowed to stand in the cold for a few hours, filtered to remove a small amount of brown precipitate, kept at 50°C. in a vacuum oven for 24 hours, and finally dried completely in a vacuum desiccator. During the drying the material is stirred occasionally to break up the caking.

Since guanidine carbonate is not stable indefinitely at 50°C. if the amount of solution being dried is too great to be handled by the vacuum oven in 24 hours, either the solution is evaporated in successive small portions or the solution is first evaporated to a thick suspension on an electric hot plate. The solution heated on the hot plate is placed in a Pyrex Top of the Oven frying pan, is stirred with an L shaped glass stirrer, and a blast

of air from a strong fan is directed on the solution. Under these conditions rapid evaporation takes place without the temperature going above 50°C., or during most of the evaporation above 37°C. This procedure is extraordinarily simple and effective.

The Nitroprusside Test.—The nitroprusside test in guanidine hydrochloride solution is carried out as follows. To 0.5 cc. of 1 or 2 per cent egg albumin there are added 2 drops of neutral 1.0 M phosphate, and 0.7 gm. guanidine hydrochloride. The tube containing the solution is placed in 37°C. water for 2–3 minutes and then in ice water. After the solution has been cooled, there are added 1 drop of 5 per cent sodium nitroprusside and 1 drop of 27 per cent ammonia. 0.5 cc. of 2 per cent egg albumin gives, within a third, as strong a color as 0.5 cc. of 0.002 M cysteine. The conditions for a quantitative nitroprusside test have not been worked out.

A little cyanide can be added to combine with traces of heavy metal compounds. 1 drop of 0.1 N KCN or NaCN is added before the addition of guanidine. No nitroprusside test is obtained in the presence of this small amount of cyanide with cystine or egg albumin whose SH groups have been oxidized to S-S groups by the addition of ferricyanide in guanidine hydrochloride solution, as described in a later section.

The S-S form of egg albumin gives a nitroprusside test when strong cyanide is added which can reduce S-S to SH. To a guanidine hydrochloride solution of the S-S protein, 1 drop of 2 N NaCN and 1 drop of ammonia are added, the solution is allowed to stand 5 minutes at room temperature and is then cooled in ice water. On the addition of 1 drop of nitroprusside a pink color is obtained.

If the nitroprusside test described in the first paragraph of this section is carried out in a solution of denatured egg albumin containing urea instead of guanidine hydrochloride the color obtained is much less intense than if guanidine hydrochloride is used.

If egg albumin is denatured by trichloracetic acid or Duponol PC and no guanidine hydrochloride or urea is present then only a negligible weak pink color is obtained on the addition of nitroprusside and ammonia. If the ammoniacal solution of egg albumin denatured by trichloracetic acid or Duponol PC is saturated with ammonium sulfate, the protein is precipitated and this precipitate gives a faint pink color with nitroprusside. This faint pink color is much intensified on the further addition of solid guanidine hydrochloride or solid thiocyanate.

Titrations in Guanidine Hydrochloride Solution.—First, the standard titration procedure. To 0.5 cc. of 2 per cent egg albumin there are added 0.1 cc. of buffer solution containing equal parts of 1.0 M Na_2HPO_4 and 1.0 M NaH_2PO_4, 0.5 cc. of 0.002 M ferricyanide, tetrathionate, or mercuribenzoate, and 1.2 gm. of guanidine hydrochloride of tested purity. The solution is placed in 37°C. water for 3 minutes and cooled in ice water. Then 1 drop of 5 per cent nitroprusside and 1 drop of 27 per cent ammonia are added. No pink color is observed. If 1 drop of 0.1 N NaCN is added before the nitroprusside, still no pink color is obtained. If 0.5 cc. of 0.0018 M ferricyanide, tetrathionate, or mercuribenzoate is added, then a weak pink is obtained about equal to that obtained from 0.5 cc. of 0.2 per cent albumin to which no SH reagent has been added.

If 0.5 cc. of 0.002 M ferricyanide or mercuribenzoate is used, no nitroprusside test is obtained whether the nitroprusside test is carried out as quickly as possible after the solution of the guanidine and cooling of the solution or after the solution containing protein, ferricyanide, and guanidine has stood 30 minutes at 37°C. If 0.5 cc. of 0.0018 M ferricyanide or mercuribenzoate is used a small nitroprusside test is obtained whether the test is carried out as soon as possible or after 30 minutes.

If the guanidine hydrochloride used is free of impurities which bring about the abolition of SH groups, the ferricyanide can be added 30 minutes after the guanidine hydrochloride without any change in the amount of ferricyanide needed to abolish the nitroprusside test. To 0.5 cc. of protein solution plus 0.1 cc. of phosphate buffer there is added 0.7 gm. of guanidine hydrochloride. The solution is allowed to stand 30 minutes at 37°C., 0.5 cc. of ferricyanide is added, the solution is allowed to stand 3 minutes more at 37°C. before being cooled in ice water. Finally 1 drop of nitroprusside and 1 drop of ammonia are added to find out whether the SH groups have all been oxidized.

The standard titration can be carried out with 0.5 cc. of 0.4 per cent egg albumin instead of 0.5 cc. of 2 per cent egg albumin. The less protein is used, the weaker the nitroprusside test given when only 10 per cent of the SH groups survive. Instead of titrating a dilute solution, one can concentrate the protein. A volume of egg albumin solution containing 10 mg. of protein is diluted to 9 cc. with water and 1 cc. of 2.0 N trichloracetic acid is added. The precipitate is centrifuged down and dissolved with the minimum amount of 0.5 N sodium hydroxide and the resulting solution is diluted to approximately 0.5 cc. with water. Then buffer, titrating agent, and guanidine hydrochloride are added as in the standard procedure.

Formaldehyde cannot be used as a titrating agent because it abolishes the protein SH groups only when added in excess. If 0.5 cc. of 0.004 M formaldehyde is added to the 0.5 cc. of 2 per cent egg albumin before the guanidine under the standard titration conditions, a strong positive nitroprusside is obtained. If 0.5 cc. of 38 per cent formaldehyde is added, only a slight flash of pink is observed.

Effect of Cyanide on Titrations.—The ferricyanide titration cannot be carried out in the presence of cyanide because cyanide in some way inhibits the reduction of ferricyanide by denatured egg albumin, as shown by the following experiment. 1 drop of 0.1 N NaCN is added to the protein solution before the addition of phosphate, ferricyanide, and guanidine. A strong nitroprusside test is obtained although in the absence of cyanide the nitroprusside test would be negative.

Although concentrated ferricyanide oxidizes cyanide slowly, under the conditions of the experiments just described no ferrocyanide is formed from ferricyanide by a cyanide-guanidine hydrochloride-phosphate solution which does not contain protein.

0.5 cc. of 0.002 M free cysteine in a guanidine hydrochloride-phosphate solution reduces ferricyanide in the presence as well as in the absence of 1 drop of 0.1 N NaCN. In the presence of the cyanide, however, the disappearance of the brown ferricyanide color is slow enough to be observed. In the absence of cyanide the disappearance of the brown color takes place instantaneously so far as the eye can tell.

Just as 10 mg. of denatured egg albumin in guanidine hydrochloride solution still gives a nitroprusside test after being treated with 0.5 cc. of 0.002 M ferricyanide in the presence of 1 drop of 0.1 N cyanide, so a strong nitroprusside is also obtained after treatment of 10 mg. denatured egg albumin with 0.5 cc. of 0.002 M mercuribenzoate in the presence of cyanide. Presumably cyanide combines with the heavy metal in mercuribenzoate and so prevents the mercuribenzoate from combining with SH groups. In contrast, 0.5 cc. of 0.002 M tetrathionate abolishes the nitroprusside test in the presence as well as in the absence of 1 drop of 0.1 N cyanide.

Tests for Completeness of SH Reactions in Neutral Guanidine Hydrochloride Solution.—The following series of experiments was designed to find out whether the abolition of the SH groups by the SH reagents takes place entirely in the 3 minute reaction in neutral

solution, or whether part of the abolition of SH groups takes place after ammonia is added for the nitroprusside test.

After the protein and titrating agent have been in the neutral guanidine hydrochloride solution for 3 minutes at 37°C. under the conditions of the standard titration water is added to 9 cc. and then 1 cc. of 2.0 N trichloracetic acid. The precipitate is centrifuged, washed with 0.2 N trichloracetic acid, and centrifuged again. Water is added to the precipitate to make the volume approximately 1 cc. (previously marked on the tube) and the precipitate is dissolved with 1 gm. of guanidine hydrochloride and cooled in ice water. Then nitroprusside and ammonia are added. The protein treated with ferricyanide for 3 minutes gives no nitroprusside test, the protein treated with tetrathionate a moderately strong test, the protein treated with mercuribenzoate about as strong a test as untreated protein. The experiments are repeated, trichloracetic being added after tetrathionate and mercuribenzoate have stood in the neutral protein-guanidine solution for 30 minutes instead of 3 minutes. This time the protein treated with tetrathionate gives no nitroprusside test (even if 1 drop of 0.1 N cyanide is present during the tetrathionate reaction) but the protein treated with mercuribenzoate still gives as strong a test as before. Thus the reaction between ferricyanide and the SH groups of denatured egg albumin in neutral guanidine solution (before the addition of ammonia) is completed in 3 minutes, whereas the reaction with tetrathionate is completed in 30 minutes but not in 3 minutes.

The fact that the trichloracetic acid precipitate of albumin treated with mercuribenzoate gives a nitroprusside test shows that the compound between mercuribenzoate and the protein SH groups must be dissociated by trichloracetic acid, for the following experiment shows that mercuribenzoate actually does combine with the SH groups of denatured egg albumin in neutral solution. After the protein solution containing guanidine hydrochloride and mercuribenzoate has stood 3 minutes, 2 drops of 0.01 M ferricyanide are added. The protein after being precipitated and washed with trichloracetic acid still gives a strong nitroprusside test in guanidine solution. If mercuribenzoate is omitted the nitroprusside test is abolished by ferricyanide. Thus mercuribenzoate prevents the oxidation of the SH groups by ferricyanide in neutral guanidine solution.

Ferricyanide Reduction Test for SH Groups.—The SH groups of the trichloracetic acid precipitate of protein treated with ferricyanide or tetrathionate in guanidine hydrochloride solution can also be measured by the ferricyanide-Duponol PC method. The results confirm those obtained by the nitroprusside test.

The washed trichloracetic acid precipitate is dissolved by the addition of 0.5 cc. of 10 per cent Duponol PC, 0.3 cc. of 0.5 N NaOH (to neutralize the trichloracetic acid), and 0.2 cc. of the neutral phosphate buffer. 0.5 cc. of 0.1 M ferricyanide is added and after the solution has been in a 37°C. bath for 10 minutes the ferrocyanide formed is measured as Prussian blue as described in the following section. The proteins treated with ferricyanide for 3 minutes or with tetrathionate for 30 minutes give no ferrocyanide just as they give no nitroprusside test. The protein treated with tetrathionate for 3 minutes gives the equivalent of 1 cc. of 0.0006 M ferrocyanide just as it gives a moderate nitroprusside test. Protein originally treated with 1 cc. of 0.0008 M ferricyanide gives 1 cc. of 0.00016 M ferrocyanide just as it gives a small nitroprusside test.

The procedure just described can be used to test the effect of cyanide on the oxidation of the SH groups of egg albumin by ferricyanide and tetrathionate. 10 mg. of denatured egg albumin in guanidine hydrochloride solution are exposed for 30 minutes to 0.5 cc.

of 0.002 M ferricyanide or tetrathionate in the presence of 1 drop of 0.1 cyanide. The protein is then precipitated and washed with trichloracetic acid, dissolved in neutral Duponol PC solution, and the surviving SH groups measured with ferricyanide. Although in the absence of cyanide all the SH groups are abolished, in the presence of cyanide ferricyanide abolishes only 55 per cent of the SH groups, tetrathionate 95 per cent. This confirms the result obtained by the nitroprusside test that cyanide interferes with the ferricyanide reaction more than it interferes with the tetrathionate reaction.

Ferricyanide Reduction in Guanidine Hydrochloride Solution.—To 0.5 cc. of 5 per cent egg albumin there are added 2 drops of phosphate buffer, 1 drop of 0.1 or 0.5 M ferricyanide, and 0.6 gm. guanidine hydrochloride. After the solution has been kept in a 37°C. water bath for 3 minutes there are added 1 cc. of water, 0.5 cc. of 2.0 N sulfuric acid, 18 cc. of water, and 2.5 cc. of 2.0 N trichloracetic acid. The suspension is well mixed and centrifuged. If any particles remain in the supernatant solution, the centrifuging is repeated. Filtration results in some loss of ferrocyanide. To 9 cc. of the supernatant solution there are added 0.5 cc. of 0.1 M ferricyanide and 0.5 cc. of ferric sulfate solution. After 20 minutes the Prussian blue is read in the light transmitted by a red filter against the Prussian blue developed from 1 cc. of 0.0025 M ferrocyanide. The two color values agree within 5 per cent. The ferrocyanide standard is made up as follows. To 0.5 cc. of 5 per cent egg albumin there are added 2 drops phosphate solution and 0.6 gm. guanidine hydrochloride. After the solution has stood at 37°C. for 3 minutes 1 cc. of 0.0025 M ferrocyanide is added. Then sulfuric acid and the other reagents are added as before to develop the Prussian blue.

Since guanidine hydrochloride in sufficient concentration interferes with the development of Prussian blue, the experiment is arranged so as to keep the concentration of guanidine hydrochloride as low as possible.

Guanidine hydrochloride, unlike Duponol PC, does not prevent the precipitation of denatured egg albumin by acid ferric sulfate. That is why the protein is precipitated with trichloracetic acid and removed before the addition of ferric sulfate. Duponol PC cannot be added to the acid solution to keep the protein in solution because it forms a precipitate with guanidine hydrochloride.

Ferricyanide Reduction in Urea Solution.—In one test tube there are added to 0.5 cc. of 2 per cent egg albumin 2 drops of 1 N hydrochloric acid and 0.6 gm. urea. After this test tube has been at 37°C. for 5 minutes there is added from another test tube a mixture of 0.5 cc. 0.002 M or 0.1 M ferricyanide, 2 drops 1 N sodium hydroxide, 4 drops 1 M neutral phosphate, and 0.8 gm. urea. After 5 minutes more at 37°C. the reaction is stopped by 0.5 cc. of 2 N sulfuric acid and then there are added 0.5 cc. of 1 M ferricyanide (only to the solution containing 0.5 cc. of 0.002 M ferricyanide), water to 9.5 cc., and 0.5 cc. ferric sulfate solution. The Prussian blue formed is equivalent to 1 cc. of 0.001 M ferrocyanide, within 5 per cent, whether 0.002 M or 0.1 M ferricyanide is originally added.

Titrations in Duponol PC Solution.—First the mercuribenzoate titration. To 1 cc. of 1 per cent egg albumin are added 2.3 cc. of water, 0.2 cc. of the neutral 1.0 M phosphate, and 0.5 cc. of 10 per cent Duponol PC. The solution is brought to 37°C. and 0.5 cc. of 0.02 M ferricyanide is added. After the solution has been at 37°C. for 1 minute there are added 0.5 N of 2.0 N sulfuric acid, water to 9.5 cc., and 0.5 cc. of ferric sulfate solution. After 20 minutes the Prussian blue developed is estimated colorimetrically in the light transmitted by a red filter. The Prussian blue is the same, within 5 per cent, as that developed from 1 cc. of 0.001 M ferrocyanide (*cf.* Anson, 1939). If 1 cc. of 0.001 M

mercuribenzoate is added to the protein-Duponol solution before the ferricyanide and the solution is allowed to stand 1 minute at 37°C. before the addition of ferricyanide, then no Prussian blue is obtained. Thus mercuribenzoate prevents the reduction of ferricyanide by denatured egg albumin in Duponol PC as in guanidine hydrochloride solution. In practice, to avoid the difficulty of estimating very weak colors, 1 cc. of 0.001 M ferrocyanide is added after the reaction has been stopped with sulfuric acid. The Prussian blue obtained is that expected from 1 cc. of 0.001 M ferrocyanide. If 1 cc. of 0.0008 M mercuribenzoate is used instead of 0.001 M mercuribenzoate, then on the addition of 1 cc. of 0.001 M ferrocyanide as much Prussian blue is developed (to quote a single experiment) as would be developed from 1 cc. of 0.00115 M ferrocyanide. The 0.0008 M mercuribenzoate does not completely abolish the SH groups and so some ferricyanide is reduced.

A control experiment is carried out to show that under the conditions used mercuribenzoate does not interfere with the estimation of ferrocyanide. 1 cc. of 0.001 M ferrocyanide is added after the mercuribenzoate and the ferricyanide is added after the sulfuric acid which stops all reduction of ferricyanide. The Prussian blue formed is that expected from the amount of ferrocyanide added. If, however, the solution containing mercuribenzoate and ferrocyanide is allowed to stand 5 minutes (instead of 1 minute as in the actual experiment) before the addition of acid ferric sulfate, then less than the expected amount of Prussian blue is obtained. Presumably in the presence of mercury salt and air some ferrocyanide is oxidized.

Another control experiment shows that under the conditions used mercuribenzoate does not reduce ferricyanide. After the ferricyanide has been allowed to react with denatured egg albumin in the absence of mercuribenzoate, 1 cc. of 0.001 M mercuribenzoate is added and the solution is allowed to stand 1 minute before the addition of acid and ferric sulfate. The amount of Prussian blue obtained is the same as that obtained when mercuribenzoate is not added.

For the ferricyanide and tetrathionate titrations in Duponol PC solution the nitroprusside test is used for the end point. To 0.5 cc. of 2 per cent egg albumin are added 0.2 cc. phosphate solution, 0.5 cc. of 0.002 M ferricyanide or tetrathionate, and 0.5 cc. of 0.8 per cent Duponol PC solution. After this solution has stood 10 minutes in the ferricyanide titration and 30 minutes in the tetrathionate titration water is added to 9 cc., the protein is precipitated by the addition of 1 cc. 2.0 N trichloracetic acid and warming of the solution to 60°C., washed with 0.2 N trichloracetic acid, diluted to 1 cc., and dissolved with 1 gm. of guanidine hydrochloride. No pink color is obtained on the addition of nitroprusside and ammonia. If 10 per cent less ferricyanide or tetrathionate is used, a weak color is obtained in the nitroprusside test.

Only 4 mg. of Duponol PC is used in the experiment just described because larger amounts of Duponol interfere with the precipitation of the protein with trichloracetic acid. 10 mg. of Duponol PC does not prevent the precipitation of the ordinary SH form of egg albumin, but it prevents the precipitation by trichloracetic acid if the protein SH groups are first oxidized to S-S groups.

A control experiment shows that even when only 4 mg. of Duponol PC is used to denature the egg albumin, all the 0.5 cc. of 0.002 M ferricyanide added is reduced to ferrocyanide. After the ferricyanide has reacted with the denatured egg albumin in neutral Duponol solution, there are added 0.5 cc. of 2.0 N sulfuric acid, 0.5 cc. of 10 per cent Duponol PC, 0.5 cc. of 0.1 M ferricyanide, water to 9.5 cc., and 0.5 cc. of the ferric

sulfate solution. As much Prussian blue is formed, within 5 per cent, as from 1 cc. of 0.001 M ferrocyanide. Extra Duponol is added after the reaction has been stopped by sulfuric acid to prevent the precipitation of protein by acid ferric sulfate. Extra ferricyanide is added to speed up the formation of Prussian blue in Duponol solution.

Cyanide inhibits the reduction of ferricyanide by denatured egg albumin in Duponol solution even more than it does in guanidine hydrochloride solution. If in the experiment just described 1 drop of 0.1 N cyanide is added before the addition of Duponol and the ferricyanide is in contact with the albumin 30 minutes, only 1 cc. of 0.00007 M ferrocyanide is formed. In contrast 0.5 cc. of 0.002 M tetrathionate abolishes the nitroprusside test of 10 mg. of denatured albumin even if 1 drop of 0.1 N cyanide is present during the tetrathionate reaction.

Reactions of Iodine with Native Egg Albumin.—First, the experiments showing the minimum amount of iodine which abolishes the nitroprusside test. To 0.5 cc. of 2 per cent egg albumin are added at 0°C. 0.1 cc. of neutral 1.0 M phosphate and 0.5 cc.of 0.0026 N iodine. The solution is allowed to stand 5 minutes at 0°C., during which time all the iodine added is absorbed as shown by a negative starch test. The egg albumin treated with iodine whether dialyzed free of iodide or not gives a negative nitroprusside test in guanidine hydrochloride solution but if it is first allowed to stand in an alkaline solution containing strong cyanide, a strong positive nitroprusside test is obtained. The techniques of the nitroprusside tests are described in the section on the nitroprusside test. If 10 per cent less iodine is used a weak positive nitroprusside test is obtained without the preliminary treatment with alkaline cyanide.

In the next experiments more iodine is added at 37°C., and there are no surviving S-S groups which give the nitroprusside test after exposure to alkaline cyanide and no uniodinated tyrosine groups which give the Millon test. To 0.5 cc. of 2 per cent egg albumin there are added 0.1 cc. of phosphate buffer and 0.5 cc. of 0.08 N iodine. The solution is allowed to stand 2 hours at 37°C. Then water is added to 9 cc. and 1 cc. of 2.0 N trichloracetic acid. The precipitate is centrifuged, stirred up with 10 cc. of 0.2 N trichloracetic acid, and centrifuged again. The precipitate is suspended in enough water to make the volume approximately 1 cc., and is dissolved with 1.2 gm. guanidine hydrochloride, and 1 drop of 2.0 N cyanide and 1 drop of ammonia are added. After 5 minutes 1 drop of 5 per cent nitroprusside is added. No pink color is obtained. A Millon test is carried out on the 0.5 cc. of the precipitate washed with trichloracetic acid by adding 3 drops of Millon's reagent and heating in boiling water for 2–3 minutes. No purple color is obtained. The Millon reagent used is made up as follows. 10 gm. of mercury are digested in 20 gm. of nitric acid of specific gravity 1.42 until NO_2 no longer comes off. The solution is diluted with twice its volume of water and stored in a brown bottle. In carrying out the Millon test it is necessary to heat long enough to bring out the full color and not long enough to make the color disappear again.

If 0.06 N iodine is added to 37°C. in the experiment just described, instead of 0.08 N iodine, the protein after being precipitated with trichloracetic acid gives a weak cyanide-nitroprusside test and a weak Millon test.

If after the iodine is added the solution is allowed to stand 1 hour at 60°C. instead of 2 hours at 37°C., 0.5 cc. of 0.05 N iodine has to be added to abolish the cyanide-nitroprusside and the Millon test of 0.5 cc. of 2 per cent egg albumin. If 0.04 N iodine is added faint positive tests are obtained.

A control experiment is done to show that the trichloracetic precipitation and washing adequately removes the tetrathionate formed by the reaction of iodine and thiosulfate.

0.5 cc. of 0.08 N iodine and 0.5 cc. 0.08 M thiosulfate are mixed before being added to egg albumin. The protein after being precipitated and washed with trichloracetic acid gives strongly positive cyanide-nitroprusside and a strongly positive Millon test.

Mercuribenzoate Plus Native and Denatured Egg Albumin.—The following experiments show that if mercuribenzoate combines with native egg albumin at all, the compound is much looser than the compound between mercuribenzoate and denatured egg albumin.

First it is shown that under the conditions used ferricyanide is not reduced by either native egg albumin or by denatured egg albumin in Duponol solution. To 1 cc. of 1 per cent native egg albumin are added 0.2 cc. of phosphate buffer and 0.5 cc. of water. The solution is cooled to 0°C. 1 cc. of 0.001 M ferricyanide previously cooled to 0°C. is added, the solution is allowed to stand 1 minute at 0°C., and then there are added 0.5 cc. of 2.0 N sulfuric acid, 1 cc. of 0.001 M ferrocyanide, 0.5 cc. of 0.02 M ferricyanide, 0.5 cc. of 10 per cent Duponol PC, water to 9.5 cc., and 0.5 cc. ferric sulfate solution. The Prussian blue obtained is the same in amount as the Prussian blue obtained from 1 cc. of 0.001 M ferrocyanide alone, indicating no reduction of ferricyanide by native egg albumin.

The same experiment is repeated with denatured egg albumin, 0.5 cc. of 10 per cent Duponol PC being added to 1 cc. of egg albumin instead of 0.5 cc. of water and no Duponol being added after the acid. Again no ferricyanide is reduced.

The next experiments show that cysteine added to native or denatured egg albumin is free to reduce ferricyanide. 1 cc. of cold 0.001 N cysteine (in 0.01 N hydrochloric acid) is added to the cold native or denatured egg albumin, the ferricyanide added as promptly as possible after the cysteine, and the acid is added 1 minute later. The equivalent of 0.75 – 0.9 cc. of ferrocyanide is formed. Some cysteine is unavoidably oxidized by the oxygen of the air and the amount oxidized is variable.

Finally, the experiments which show whether or not added mercuribenzoate is free to combine with cysteine. 1 cc. of 0.001 M mercuribenzoate is added to the native or denatured egg albumin before the solution is cooled and cysteine and ferricyanide are added. In the solution of native egg albumin no ferrocyanide is formed, showing that the mercuribenzoate has combined with the cysteine and that the cysteine-mercuribenzoate compound does not reduce ferricyanide. In the solution of denatured egg albumin, however, the same amount of ferrocyanide is formed from ferricyanide as in the absence of mercuribenzoate, showing that the mercuribenzoate has combined with the protein and is not removed from the protein by the cysteine which remains free to reduce ferricyanide.

If the experiment in Duponol solution is repeated with 1 cc. of water substituted for the 1 cc. of protein solution, no ferrocyanide is formed, showing that mercuribenzoate can combine with the cysteine in the Duponol solution if the mercuribenzoate is not combined with protein.

The Mercuribenzoate-Cysteine Compound.—If 1 cc. of 0.001 M cysteine is added to a neutral phosphate solution containing 1 cc. of 0.0001 M mercuribenzoate, the resulting solution does not give a nitroprusside test but does immediately decolorize 1 cc. of 0.001 N iodine solution.

SUMMARY

1. 1 cc. of 0.001 M ferricyanide, tetrathionate, or *p*-chloromercuribenzoate is required to abolish the SH groups of 10 mg. of denatured egg albumin in

guanidine hydrochloride or Duponol PC solution. Both the nitroprusside test and the ferricyanide reduction test are used to show that the SH groups have been abolished.

2. 1 cc. of 0.001 M ferrocyanide is formed when ferricyanide is added to 10 mg. of denatured egg albumin in neutral guanidine hydrochloride or urea solution. The amount of ferricyanide reduced to ferrocyanide by the SH groups of the denatured egg albumin is, within wide limits, independent of the ferricyanide concentration.

3. Ferricyanide and *p*-chloromercuribenzoate react more rapidly than tetrathionate with the SH groups of denatured egg albumin in both guanidine hydrochloride solution and in Duponol PC solution.

4. Cyanide inhibits the oxidation of the SH groups of denatured egg albumin by ferricyanide.

5. Some samples of guanidine hydrochloride contain impurities which bring about the abolition of SH groups of denatured egg albumin and so interfere with the SH titration and the nitroprusside test. This interference can be diminished by using especially purified guanidine hydrochloride, adding the titrating agent before the protein has been allowed to stand in guanidine hydrochloride solution, and carrying out the nitroprusside test in the presence of a small amount of cyanide.

6. The SH groups of egg albumin can be abolished by reaction of the native form of the protein with iodine. It is possible to oxidize all the SH groups with iodine without oxidizing many of the SH groups beyond the S-S stage and without converting many tyrosine groups into di-iodotyrosine groups.

7. *p*-chloromercuribenzoate combines with native egg albumin either not at all or much more loosely than it combines with the SH groups of denatured egg albumin or of cysteine.

8. The compound of mercuribenzoate and SH, like the compound of aldehyde and SH and like the SH in native egg albumin, does not give a nitroprusside test or reduce ferricyanide but does reduce iodine.

REFERENCES

Anson, M. L., 1939, *J. Gen. Physiol.*, **23**, 239.

Anson, M. L., 1940 *a*, *J. Gen. Physiol.*, **23**, 321.

Anson, M. L., 1940 *b*, *J. Biol. Chem.*, **135**, 797.

Arnold, V., 1911, *Z. physiol. Chem.*, **70**, 300, 314.

Bernheim, F., and Bernheim, M., 1939, Cold Spring Harbor symposia on quantitative biology, Cold Spring Harbor, Long Island Biological Association, **7**, 174.

Folin, O., and Malmros, H., 1929, *J. Biol. Chem.*, **83**, 115.

Greenstein, J., 1938, *J. Biol. Chem.*, **125**, 501.

Harrington, C. R., and Neuberger, A., 1936, *Biochem. J.*, London, **30**, 809.
Heffter, A., 1907, *Med. Naturwissenschaft. Arch.*, **1**, 81. *Chem. Z.*, **11**, 822.
Hellerman, L., 1937, *Physiol. Rev.*, **17**, 454.
Hellerman, L., 1939, Cold Spring Harbor symposia on quantitative biology, Cold Spring Harbor, Long Island Biological Association, **7**, 165.
Herriott, R. M., 1937, *J. Gen. Physiol.*, **20**, 335.
Kuhn, R., and Desnuelle, P., 1938, *Z. physiol. Chem.*, **251**, 14.
Mirsky, A. E., and Anson, M. L., 1935, *J. Gen. Physiol.*, **18**, 307.
Mirsky, A. E., and Anson, M. L., 1936, *J. Gen. Physiol.*, **19**, 451.
Peters, J. P., and Van Slyke, D. D., 1932, Quantitative clinical chemistry, Baltimore, The Williams & Wilkins Co., **2**, 33.
Rosenthal, S. M., and Voegtlin, C., 1933, *Pub. Health Rep.*, *U. S. P. H. S.*, **48**, 347.
Schubert, M. P., 1936, *J. Biol. Chem.*, **114**, 341.
Vaubel, W., 1900, *Z. angew. Chem.*, **13**, 1125.
Walker, E., 1925, *Biochem. J.*, London, **19**, 1082.
Whitmore, F. C., and Woodward, G. E., 1932, Organic syntheses, Collective volume 1, New York, John Wiley and Sons, Inc., 153.

ENZYMES IN ONTOGENESIS (ORTHOPTERA)

XIV. The Action of Proteins on Certain Activators of Protyrosinase*

By JOSEPH HALL BODINE and LOREN D. CARLSON

(*From the Zoological Laboratory, State University of Iowa, Iowa City*)

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INTRODUCTION

The enzyme tyrosinase can be obtained from the egg of the grasshopper, *Melanoplus differentialis*, in an inactive form, designated protyrosinase (Bodine, Allen, and Boell, 1937; Allen, Ray, and Bodine, 1938). Protyrosinase may be activated by a number of surface active compounds including a naturally occurring substance found in the lipoidal layer of a centrifuged egg brei (Bodine, Allen, and Boell, 1937; Bodine and Allen, 1938 *a* and 1938 *b*). It may also be converted to the active form (tyrosinase) by heat (Bodine and Allen, 1938 *b*), by acetone, chloroform, urea, and urethane (Bodine and Allen, 1938 *a*, 1938 *b*), and by dialysis against distilled water (Ray and Bodine, 1939). Furthermore, it has recently been shown that some constituents in the egg brei inhibit the activating power of certain of these activators (natural activator, sodium oleate, Duponol, Aerosol) presumably by forming films over the surface of the activator micelles (Bodine and Carlson, 1940). It is not unreasonable to assume that this inhibition of the activators is due to proteins which are normal constituents of the brei since proteins have been shown to form films on quartz or collodion particles placed in their solutions (Moyer and Moyer, 1940) as well as on oil droplets found in living forms (Danielli and Harvey, 1934; Danielli, 1935).

It is the purpose of the following to show that inhibition of the various activators may be accomplished by pure protein solutions and that the inhibition may be enhanced by higher temperatures. Since the effect of heat suggests a possible relation to a denaturation, exposures of these activator-protein complexes to ultraviolet light were also made previous to the application of heat.

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Material and Methods

The protyrosinase employed was similar to that previously described and designated as B_1 (Bodine *et al.*, 1939). The activators used were sodium oleate (Merck) and Aerosol (American Cyanamid). Protein solutions were made by placing 1 gm. of the protein in 99 cc. of distilled water. This was allowed to stand and then filtered. In the case of egg albumin the stock solution was 1 per cent; in the case of hemoglobin, the solution was diluted to 0.2 per cent. Casein and edestin are very sparingly soluble and hence their concentrations in final solutions were difficult to evaluate. The brei minus AC was prepared in the same manner as that already described (Bodine and Carlson, 1940).

Temperature exposures were accomplished by placing a test tube containing the protein and activator in a water bath kept at the required temperature for a period of 10 minutes after which the tube was removed and cooled to 25°C. or below as rapidly as possible. Concentrations of activators were chosen so as to fall on or below the critical or maximum point of activation for the amount of enzyme employed (see Bodine and Carlson, 1940).

The source of ultraviolet light was the entire spectrum of an air-cooled quartz mercury vapor lamp operating on 110–115 volts A.C. at 3 amperes. The solutions were placed at a distance of 15 cm. from the light source in glass dishes covered with quartz plates $2\frac{1}{2}$ mm. thick (controls with glass). The dishes were placed on damp towelling or in a water bath of the desired temperature. The length of exposure in these experiments was 20 minutes.

The effects of these various substances and treatments were checked by testing the ability of the activator to convert protyrosinase to tyrosinase. The amount of active enzyme formed was determined in standard Warburg manometers at 25°C. using a known amount of tyramine-HCl as a substrate. The activity is expressed as $1/T \times 10^3$ where T is the time required to oxidize one-half of the substrate.

RESULTS

The addition of increasing amounts of protein to sodium oleate greatly decreases or inhibits the latter's activating potencies for protyrosinase with the exception of egg albumin which produces relatively slight inhibition at room temperature (Fig. 1). If, however, egg albumin is heated with sodium oleate at 80°C. for 10 minutes, a marked inhibiting action is then observed (Fig. 1). The relative effectiveness of the proteins used in producing this inhibition of the activating properties of sodium oleate is clearly indicated in Fig. 1. When B_1 (protyrosinase) is first mixed with the sodium oleate and the protein then added, no inhibition occurs except for a very slight effect in the case of casein. Upon the simultaneous addition of two proteins to the activator, previous to the B_1 addition, protyrosinase is always activated less than if the second protein were not present. Slight additive effects of the proteins seem evident but are rather difficult to evaluate.

As increasing amounts of protyrosinase are added to a constant amount of sodium oleate an increasing rate of oxidation of the substrate results (Bodine and Allen, 1938 *b*). The possible mechanism of this activation has been previously described (Bodine and Carlson, 1940) as being the adsorption or orientation of the proenzyme on oleate micelles. If one assumes that the proenzyme is protein in nature, and there seems good evidence for such an assumption, the relation between the activator and other proteins may be the same as that between protyrosinase and sodium oleate. To

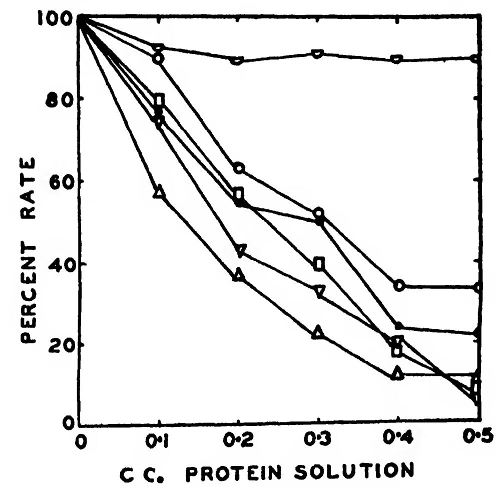

FIG. 1. The effect of varying amounts of protein solutions on the activating power of a constant amount of sodium oleate. Abscissa, amounts of solution added; ordinate, per cent of rate given with sodium oleate alone. ▽, 1 per cent albumin; O, 1 per cent albumin heated 80°C. for 10 minutes with the sodium oleate; ●, 0.2 per cent hemoglobin; □, casein; △, edestin; ▽, obverse curve of protyrosinase activation curve. The values shown on the abscissa are one-half actual value for the protyrosinase curve.

illustrate this an obverse curve of the activity of increasing amounts of proenzyme in relation to a constant amount of activator is shown in Fig. 1. This curve is the reverse of what would actually be shown if the activity of the mixture were placed on the ordinate and demonstrates that while certain proteins remove the potential activating power of sodium oleate, protyrosinase does essentially the same thing.

The effect of heat on activator-protein complexes was tested by taking a ratio of protein to activator which, as the result of experimental tests, showed moderate inhibition and heating this mixture and then rechecking its activating capacity. Hemoglobin and casein in such systems are unaffected by temperatures up to 80°C. (Fig. 2). The effect of different

temperatures (25–80°) on albumin and edestin is shown in Fig. 2. It will be noted that for each of these proteins there is little effect of temperature until a certain point is reached when there is a rapid increase in inhibition of the activator (compare with results for brei minus Ac—Fig. 4). This occurs between 55 and 70° for albumin and between 45 and 60° for edestin. It should be emphasized again that these effects are not obtained unless the activator and protein are heated together (see previous paper on heat and brei (Bodine and Carlson, 1940)).

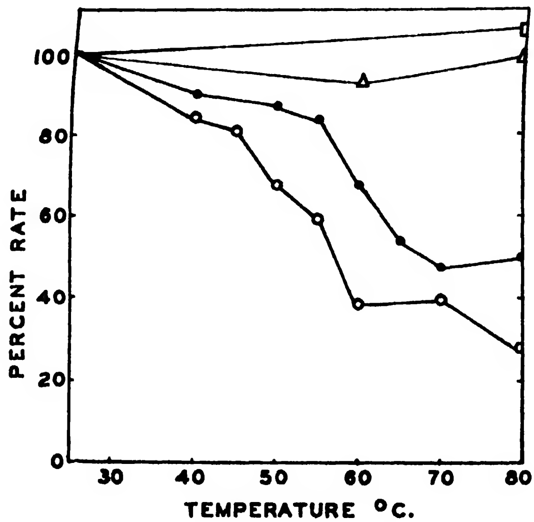

FIG. 2. The effect of temperature on mixtures of proteins and sodium oleate. Abscissa, temperature in degrees C.; ordinate, the per cent rate in terms of the mixtures at 25°C. ●, albumin with sodium oleate; ○, edestin with sodium oleate. △, casein; □, hemoglobin. Concentrations of proteins constant for any protein throughout experiments and chosen so that rate changes could be easily followed.

Inasmuch as protyrosinase (B_1), a protein, can in itself be activated by temperature (Bodine and Allen, 1938 *b*) it becomes of some interest, in light of the above results, to determine the action of heat on it in the presence of sodium oleate. The addition of a small amount of sodium oleate to protyrosinase lowers the temperature at which heat activation becomes evident (Fig. 3). This change in the effect of temperature is in accord with the results obtained when other proteins are similarly treated with sodium oleate. It is known that the number of micelles increases with increase in temperature (Hartley, 1936). Yet the nature of the increase in degree of activation at different temperatures as well as the fact that varying amounts of sodium oleate show the same relationships seems to indicate

that the explanation of this phenomenon is not simply an increase in micelles. Moreover, mixtures of B_1 with an excess of sodium oleate that have been heated at 90° for 10 minutes show no activity when more B_1

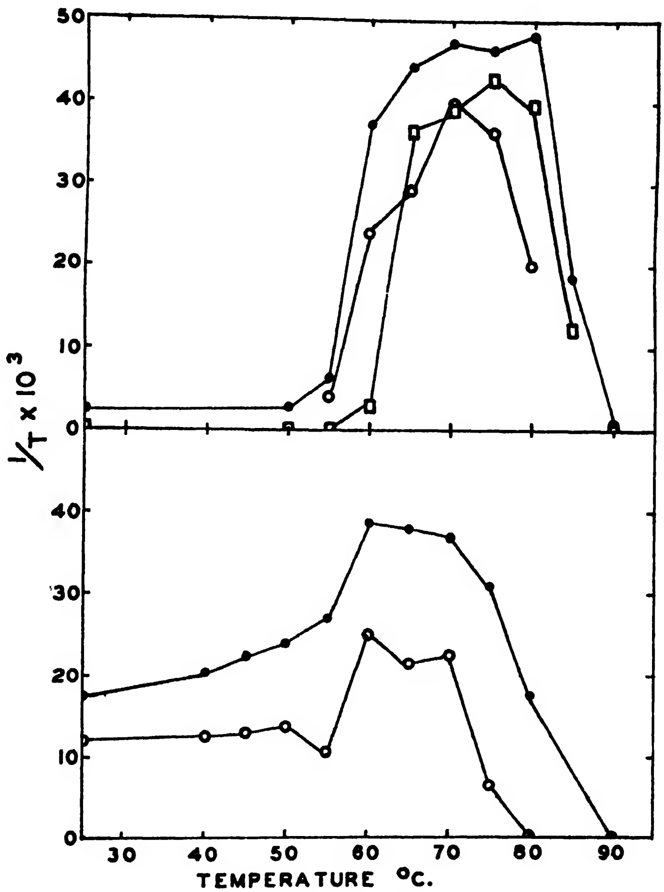

FIG. 3. The effect of temperature with and without ultraviolet light on mixtures of protyrosinase and sodium oleate. Abscissa, temperature in degrees C.; ordinate, rate of the reaction. □, the effect of heat on protyrosinase alone; ●, the effect of heat on protyrosinase in combination with a small amount of sodium oleate; ○, the effect of heat following treatment with ultraviolet light on the same combinations. Top graph shows effect with less activation by oleate than in bottom graph.

is added, indicating that the oleate-enzyme complex maintains and is irreversible or even increased in strength after such exposure to temperature; *i.e.*, the oleate is "covered over" and no longer is capable of activating protyrosinase.

It has previously been noted that all of the agents activating protyrosinase

also act upon proteins, denaturing them (Bodine and Allen, 1938 *b*). The effect of temperature on brei proteins as well as edestin and albumin in the presence of sodium oleate suggests a possible relation to denaturation. Since ultraviolet light is known to lower the temperature of protein denaturation (Stedman and Mendel, 1926; Clark, 1935; Bovie, 1913), these

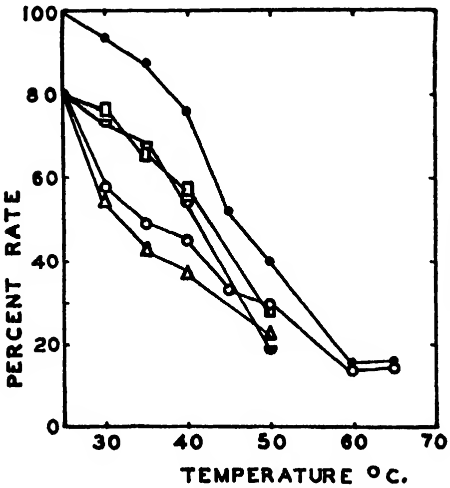

FIG. 4

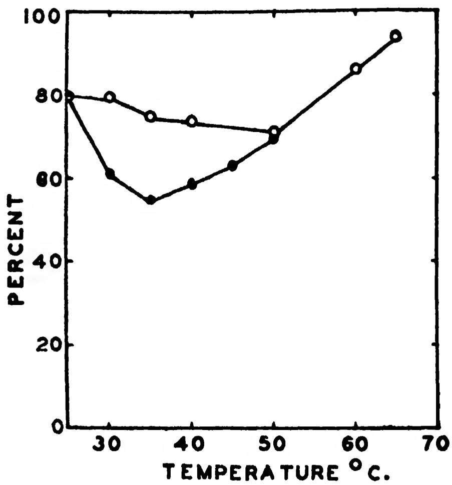

FIG. 5

FIG. 4. The effect of ultraviolet light and heat on brei minus AC with sodium oleate. Abscissa, temperatures to which the mixtures were exposed; ordinate, per cent of the rate at 25°C. ●, the effect of heat alone; ○, the effect of treatment with ultraviolet light preceding exposure to heat; □, the effect of heat treatment preceding ultraviolet exposure; △, the effect of simultaneous exposure to ultraviolet light and heat; ◡, the calculated summation effect of ultraviolet light and heat.

FIG. 5. The difference in the effects of ultraviolet light when used before or subsequent to heat or brei minus AC with sodium oleate. Abscissa, temperature in degrees C.; ordinate, the values for solutions treated with both ultraviolet light and heat in per cent of those treated with heat alone. ○, mixtures treated with heat and then ultraviolet light; ●, mixtures treated with ultraviolet light and then heat.

activator-protein complexes were subjected to ultraviolet light previous to, simultaneous with, and subsequent to heating. Figs. 4 and 5 show that exposure to ultraviolet alters the temperature effect on a mixture of protein and activator. It is evident in Fig. 4 that if ultraviolet light is applied before or simultaneous with heat, the potency decrease due to heat is greater. If, however, the ultraviolet light is used subsequent to heating, the effect is the same as that obtained at 25°C. The data in Fig. 4 are replotted in Fig. 5 to more clearly demonstrate this point. Combinations

of albumin or edestin with sodium oleate which are previously treated with ultraviolet are affected similarly.

When a mixture of protyrosinase and sodium oleate is irradiated with ultraviolet before subjection to temperature, the activity of the enzyme is reduced and the temperature of inactivation (complete denaturation) is lowered (Fig. 6). This effect of ultraviolet light on the proenzyme is analogous to the effect noted with albumin and edestin.

Many of the above reactions have been tested using Aerosol as the activator and some using olive oil and the results have been qualitatively

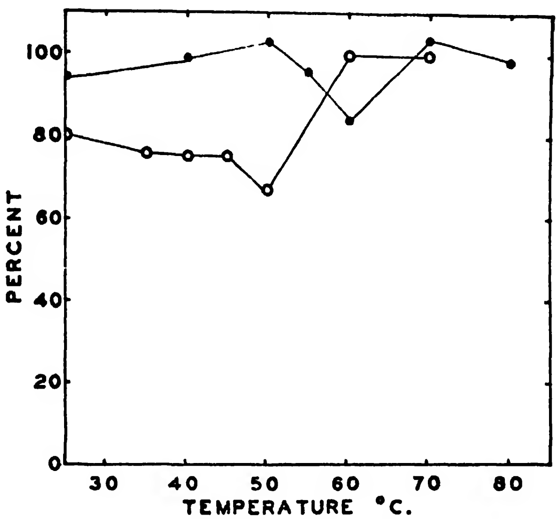

FIG. 6. The change in the heat effect on albumin or edestin with sodium oleate caused by treatment with ultraviolet. Abscissa, temperature, degrees C.; ordinate, the values for solutions treated with both ultraviolet light and heat in per cent of those treated with heat alone. ●, albumin; ○, edestin.

similar. Thus, the above mentioned conclusions seem to hold irrespective of the particular lipoidal activator.

DISCUSSION

A consideration of the above results suggests a parallelism between the mechanism of activation of protyrosinase and that for the inhibition of the activator produced by the addition of proteins, such as the brei proteins, albumin, hemoglobin, edestin, and casein. Since sodium oleate has been most extensively employed as an activator the present discussion will be based almost solely upon results obtained through its use although comparable results are produced by all activators.

The possible modes of action or the relationships between activator and protein may be looked upon in one of two ways. First, the oleate (activator) may be adsorbed on the protein. Secondly, the protein may be adsorbed on the activator micelle. (Hartley, 1936, has described these micelles for paraffin chain salts. Each micelle consists of a hydrophobic core and a hydrophilic shell.) The evidence at hand from the present work seems to more or less favor the second of the above possibilities.

Du Noüy (1926) in studying the changes in surface tension of serum upon the addition of sodium oleate concludes that the oleate is on the surface of the protein. Anson (1939) in his study of protein denaturation by detergents suggests that the denatured protein is kept in solution by detergent molecules adsorbed on their surfaces. However, he makes no mention of the mode of denaturation.

It is difficult to explain a number of the results obtained in the present experiments on the assumption that the protein adsorbs the sodium oleate molecules. Results of rather crude cataphoresis experiments show that an activator hemoglobin or activator enzyme complex moved at the same rate as the protein alone and not at a rate similar to that of oleate alone. Data from cataphoresis (Moyer, 1940; Moyer and Moyer, 1940) and surface tension (Danielli and Harvey, 1934; Danielli, 1935) methods give evidence for the adsorption of proteins on fat droplets, collodion, or quartz particles in solutions, and on fat globules in animal cells. Many of the reactions studied and especially those with heat give different results if the protein and oleate are treated together or separately. Also, if activation of the enzyme is a phenomenon similar to protein adsorption, it is difficult to conceive of it as being activated by adsorbing oleate. It seems more logical, with the evidence at hand, that it be adsorbed on the oleate micelles and thus changed in structure or configuration.

The mechanism by which the protein is adsorbed on these activator micelles is at the present not well understood. Because of the diverse types of activators and proteins it is difficult to explain adequately the relationships obtained by invoking the phenomenon of coacervation as developed by Bungenberg de Jong (1936). Micelles of oleate possess a net negative charge as well as a hydrophilic shell. At the pH (6.8) at which these studies were made hemoglobin and edestin are on the positive side of their isoelectric points. They are strongly adsorbed (Fig. 1). Albumin, negatively charged at this pH does not inhibit to a great degree until it is heated. Casein, however, with an isoelectric point similar to albumin behaves much like hemoglobin. While heat affects both edestin and albumin when they

are in contact with the activator, it has no effect on hemoglobin or casein under similar conditions. It might well be that hemoglobin and casein are completely denatured on the micelles while edestin and albumin are not. Heat then continues the denaturation process making the adsorption more complete. This seems also to be the case with mixtures of sodium oleate and the proenzyme. The changes in activity occur at temperatures below the point of heat denaturation of the protein alone. This supposition is given further support by the action of ultraviolet light on these protein-activator complexes. Exposures to ultraviolet irradiation lower the temperatures at which proteins denature (Clark, 1935; Stedman and Mendel, 1926; Bovie, 1913, etc.). Bovie (1913), repeating Chick and Martin's work on denaturation of albumin with irradiated protein, obtained a similar type of curve except that it occurred 10 to 15°C. lower than the untreated albumin. In all cases where heat affects the protein activator complexes in these experiments, ultraviolet caused a lowering of the temperature at which it occurred (Figs. 3, 5, and 6).

Activation of protyrosinase, which may be accomplished by a number of factors, seems related to a change in configuration or orientation of the protein. Northrop (1939) has shown that comparatively slight changes in an inactive protein may result in the formation of an active enzyme. In the case of the trypsinogen to trypsin conversion, the change is in the splitting of a peptide bond (Northrop, 1939).

When the activation of protyrosinase is brought about by surface active compounds, it seems to be accomplished by an adsorption on the surface of micelles of these compounds. Other proteins are adsorbed also but the adsorption of protyrosinase yields a unique configuration possessing the enzymic properties measured experimentally. This change from inactive to active enzyme may well be a mild denaturation exposing certain groups *via* opening of bonds. Other chemicals and treatments known to cause similar changes in proteins are capable of changing protyrosinase to tyrosinase.

SUMMARY

1. Proteins, when added to activators (sodium oleate, Aerosol) of protyrosinase, greatly decrease the degree of activation.

2. Certain proteins adsorbed on activator micelles are markedly affected by temperature and are rendered more sensitive by ultraviolet light.

3. Ideas are expressed as to the possible nature of activating and inhibiting phenomena especially as they relate to the enzyme tyrosinase.

LITERATURE CITED

Allen, T. H., Ray, O. M., and Bodine, J. H., *Proc. Soc. Exp. Biol. and Med.*, 1938, **39**, 549.

Anson, M. L., *J. Gen. Physiol.*, 1939, **23**, 239.

Bodine, J. H., Allen, T. H., and Boell, E. J., *Proc. Soc. Exp. Biol. and Med.*, 1937, **37**, 450.

Bodine, J. H., and Allen, T. H., *J. Cell. and Comp. Physiol.*, 1938 *a*, **11**, 409.

Bodine, J. H., and Allen, T. H., *J. Cell. and Comp. Physiol.*, 1938 *b*, **12**, 71.

Bodine, J. H., and Carlson, L. D., *J. Cell. and Comp. Physiol.*, 1940, **16**, 71.

Bodine, J. H., Ray, O. M., Allen, T. H., and Carlson, L. D., *J. Cell. and Comp. Physiol.*, 1939, **14**, 173.

Bovie, W. T., *Science*, 1913, **37**, 373.

Bungenberg de Jong, H. G., Actualités Scientifiques et Industrielles, 1936, Nos. 397–398.

Clark, J. H., *J. Gen. Physiol.*, 1935, **19**, 199.

Danielli, J. F., *J. Cell. and Comp. Physiol.*, 1935, **7**, 393.

Danielli, J. F., and Harvey, E. N., *J. Cell. and Comp. Physiol.*, 1934, **5**, 483.

Du Noüy, P. L., Surface equilibria of biological and organic colloids, New York, The Chemical Catalog Company, Inc., 1926.

Hartley, G. S., Actualités Scientifiques et Industrielles, 1936, No. 387.

Moyer, L. S., *J. Biol. Chem.*, 1940, **133**, 29.

Moyer, L. S., and Moyer, E. Z., *J. Biol. Chem.*, 1940, **132**, 357.

Northrop, J. H., Crystalline enzymes, New York, Columbia University Press, 1939.

Ray, O. M. and Bodine, J. H., *J. Cell. and Comp. Physiol.*, 1939, **14**, 43.

Stedman, H. L., and Mendel, L. B., *Am. J. Physiol.*, 1926, **77**, 199.

THE INFLUENCE OF AGE, HYPOPHYSECTOMY, THYROIDECTOMY, AND THYROXIN INJECTION ON SIMPLE REACTION TIME IN THE RAT

By E. BLOOR BRODY

(From the Departments of Dairy Husbandry and Psychology, University of Missouri, Columbia)*

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INTRODUCTION

As an organism ages, the speed of physiologic processes decreases; or, as Carrel has put it, there is a retardation of the rate of flow of physiologic time. This retardation may be attributed in large part to changes taking place within those organismic devices, such as the nervous, circulatory, and endocrine systems, which are concerned with the integration and regulation of the various specialized organs and systems. The speed of a simple reaction to an electric shock, involving as it does these integrative and conductive systems, may be thought of as dependent upon their physiologic ages, and consequently upon the rate of flow of physiologic time within the organism. This report presents data on the relationship of the speed of simple reaction to an electric shock to age, thyroidectomy, hypophysectomy, and thyroxin injection. Thyroidectomy and hypophysectomy are assumed to decrease, and thyroxin injection to increase the rate of flow of physiologic time.

Methods

For the measurement of reaction time a Dodge pendulum-photochronograph[1] was adapted for use with small animals in a manner similar to one employed by Munn.[2]

* The reaction rates were measured in the Department of Psychology; the metabolism measurements and related work were done in the Department of Dairy Husbandry. Grateful acknowledgments are made to Dr. F. A. Courts, Department of Psychology, for collaboration in adapting the Dodge pendulum-photochronograph for use in measuring reaction time in rats, and to Mr. W. W. Heathman, Department of Dairy Husbandry, for collaboration in hypophysectomy.

[1] Dodge, R. A., A pendulum-photochronograph, *J. Exp. Psychol.*, 1926, **9**, 156. Hilgard, E. R., Conditioned eyelid reactions to a light stimulus based on the reflex wink to sound, *Psychological Monographs*, 1931, **41**, No. 1.

[2] Munn, N. L., Developmental psychology, Houghton-Mifflin, New York, 1938, p. 107.

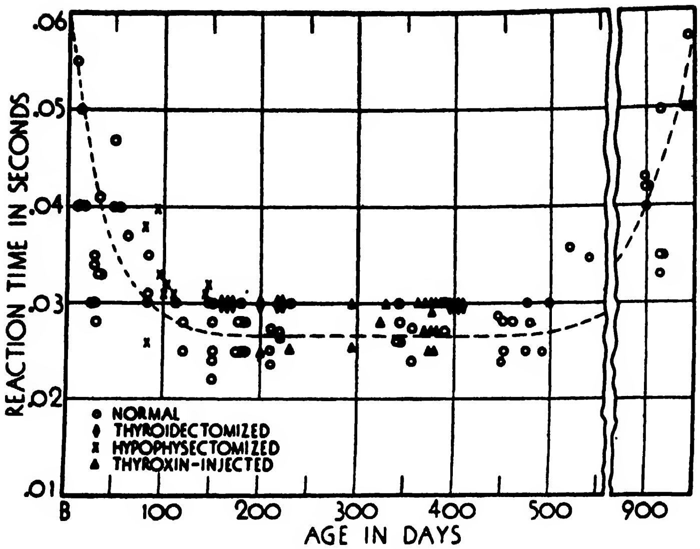

FIG. 1. Reaction rate as function of age in normal (circles), hypophysectomized (crosses), thyroidectomized (diamonds), and thyroid-injected (triangles) white rats.

TABLE I

Deviations from the Normal of Metabolic Rates of Hypophysectomized, Thyroidectomized, and Thyroxin-Injected Rats

Per cent deviation from normal

Injected with thyroxin	Hypophysectomized	Time since hypophysectomy	Thyroidectomized (some partially)	Time since thyroidectomy
		days		
+6.0	−10.3	19	−5.3	10 mos.
+13.4	−17.2	"	−9.0	" "
+13.7	−19.8	"	−9.2	" "
+18.0	−23.3	"	−26.4	" "
+24.6	−30.8	"	−11.9	25 days
+25.1	−43.4	32	−13.2	" "
+32.0	−71.9*	"	−26.5	" "
+33.2	−35.8	37	−8.6	" "
+39.6	−58.8	"	−15.1	3 mos.
+40.9				
+50.0				
+8.0				
+8.0				
+26.0				
+7.0				
+4.0				
+5.0				

* This rat died 2 days after the measurements were taken.

The animal was placed in an electrified cage mounted on tambours. The movement of the animal within the cage was recorded by the simultaneous movement of a tambour indicator. Application of the single make shock and an indicator of the moment of shock were controlled by a rotating switch tripped by a steel rod borne at right angles to the face of the pendulum bob. The source of electric energy was a storage battery, and the strength of the shock was held constant by a Harvard inductorium. The animal's feet were moistened before placing in the electrified cage. Measurements were made at a temperature of 26–30°C.

The energy metabolism rate was taken to be the index of the "rate of living" of the animals, as modified by age, thyroidectomy, hypophysectomy, and thyroxin administration. The energy metabolism was measured in an 8-chamber Regnault-Reiset metabolism apparatus, similar to the one described by Winchester.[3]

The animals were selected at random from uniform stock. Since rats may live as long as 3½ years and the oldest rats employed in the experiment were slightly over 2½ years, there was probably no selection due to age. No correlation was found between reaction time and body weight as such.

RESULTS

The influence of various factors on reaction time is indicated graphically in Fig. 1.

1. *Endocrine factors.* As indicated by Fig. 1, manipulation of the rate of flow of physiologic time (as measured by the rate of energy metabolism) by means of thyroxin injection exerted no apparent influence on the speed of reaction to an electric shock. Thyroidectomy, however, caused a very slight decrease in the speed of reaction in that the data points are consistently on the outskirts of the normal range. With one exception the same holds true for hypophysectomy. (Deviations of the metabolic rate of injected, hypophysectomized, and thyroidectomized animals from the normal are given in Table I.)

There is no doubt that the thyroid hormone not only increases general body metabolism, but the metabolism of nervous tissue as well, as indicated by an increased oxygen consumption in brain tissue of thyroid-fed rats,[4] and by a higher rate of cortical alpha rhythms in hyperthyroid humans.[5] The failure of thyroxin injection to increase the speed of reaction to an electric shock probably indicates that the conductivity of the nervous system is already at its upper limit in the normal animal. The apparent

[3] Winchester, C. F., Seasonal metabolic and endocrine rhythms in the domestic fowl, *Univ. Missouri Agric. Exp. Station Research Bull. No. 315*, 1940.

[4] Cohen, R. A., and Gerard, R. W., Hyperthyroidism and brain oxidations, *J. Cell. and Comp. Physiol.*, 1937, **10**, 223.

[5] Ross, D. A., and Schwab, R. S., The cortical alpha rhythm in thyroid disorders, *Endocrinology*, 1939, **25**, 75.

decline in reaction speed following thyroidectomy or hypophysectomy is not surprising in view of the accompanying profound decline in metabolic rate. The limiting factor in this decline in reaction speed may reside not, however, in the speed of nervous conduction, but in a lag in muscular contraction, perhaps reflecting a lowered muscular tonus.

2. *Age.* Fig. 1 shows that the reaction rate declines from birth to about 100 days of age, remains virtually constant between 100 and 500 days, and rises steeply following about 2 years of age. The decline in reaction rate between birth and 100 days may indicate the occurrence of a developmental change in certain integrative systems, probably primarily neuromuscular. The sharp rise in reaction time following age 2 years probably reflects senile changes in the conducting nervous system, since endocrine factors as such appear only very slightly to affect the reaction time. These results suggest the possibility of using reaction time as a quantitative measure of developmental rate in early life, and senescence rate at advanced age.

SUMMARY

Thyroxin injection with associated increases in metabolic rate does not significantly affect simple reaction time to an electric shock. Hypophysectomy and thyroidectomy with associated decreases in metabolic rate produce a slight decline in reaction speed. Reaction time is long in young animals, probably due to incomplete development of certain integrative and conductive systems; it remains virtually constant between puberty and relatively advanced age when it increases rapidly, probably due to physicochemical changes in the composition of the conducting nervous system.

THE RATE OF BACTERIOPHAGE INACTIVATION BY FILTRATES OF ESCHERICHIA COLI CULTURES

By EMORY L. ELLIS AND JOHN SPIZIZEN

(*From the William G. Kerckhoff Laboratories of the Biological Sciences, California Institute of Technology, Pasadena*)

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INTRODUCTION

The inactivation of bacteriophage by substances from susceptible bacteria has been studied by Burnet (1) and Freeman (2). Its inactivation by antisera has been studied by Andrewes and Elford (3) and Burnet, Keogh, and Lush (4). Recently Ashenburg *et al.* (5) reported that saline solutions of gum arabic, starch, or glycogen inactivated an anti-*Klebsiella pneumoniae* phage.

We have studied in more detail the rate of phage inactivation by culture filtrates of the susceptible bacteria in order to determine the dependence of the process on phage and inhibitor concentrations.

EXPERIMENTAL

The phage inactivating solution was prepared from culture filtrates of the susceptible strain of *coli* (B_1) previously used in this laboratory (6). The organisms were grown in synthetic medium of the composition given by Delbrück (7). With adequate aeration, these cultures attained a maximum plate count at 48 hours of more than 5×10^9 organisms per cc. 16 liter cultures were incubated for 8 days with aeration. The cells were then spun out in a Sharples supercentrifuge and the clear supernatant concentrated in vacuo at 35°C. to about one-tenth its volume. This material was then dialyzed in cellophane sausage casing against running tap water for 48 hours. The remaining solution was then clarified in the centrifuge and further concentrated in vacuo at 35°C. It was again dialyzed, clarified in the centrifuge, and passed through a Seitz filter. The final product had a pH of 6.5, contained 2.5 mg./cc. total solids, and represented a 35 to 1 concentrate of the initial filtrate of the 8 day bacterial cultures. This concentrated, dialyzed bacterial filtrate is called "filtrate" or simply "*F*" below.

Diluted bacteriophage suspensions were prepared by 100-fold dilution in distilled water of fresh filtered phage lysates of susceptible *coli* cultures. The suspension then contained 7×10^6 phage particles per cc. as determined by the plaque counting method previously described (6).

Suitable dilutions in nutrient broth of the phage inactivating filtrate were brought to temperature. To 0.9 cc. of such a dilution of *F* was added 0.1 cc. of a dilution in broth of stock phage adjusted to contain about 3×10^5 phage particles per cc. This mixture

was incubated at the selected temperature and 0.1 cc. samples removed from time to time, the amount of active phage remaining being determined by plaque counts.

Each experimental determination of active phage corresponded usually to counts of two or four plates, containing a total of 100 to 400 plaques. Thus the sampling error lies between 5 and 10 per cent. The efficiency of plating (6) may change in the course of the inactivation. The data on the later stages of the inactivation reaction cannot be interpreted quantitatively until this point is determined.

Rate of Phage Inactivation at 0°C.

A progressive decrease in phage assay was noted with all concentrations of F tried (Fig. 1). This decrease was logarithmic in all cases until 95 per cent of the phage had been inactivated, after which the reactions became slower. The reaction is therefore first order with respect to phage, as was indicated by Burnet (4) for several phages active against *B. dysenteriae.*

The rate is not proportional to the concentration of inactivator but nearly proportional to the square root of this value. The rate of phage inactivation may thus be expressed by the equation

$$-\frac{d \log (P)}{dt} = k_0(F)^{\frac{1}{2}}$$

where (P) is the phage concentration, (F) the concentration of filtrate preparation, the undiluted material being assigned the arbitrary value unity, and k_0 is a constant. Table I shows the values of k_0 calculated from this equation, using the rates obtained graphically, for inactivator concentrations from 0.001 to 0.04. At concentrations lower than these, the results become uncertain and variable.

The slower rate of inactivation of the last fraction of phage is discussed below. That this decrease in rate did not result from exhaustion of inactivator substance is shown in the experiment plotted in Fig. 2. The inactivation of phage was permitted to proceed until 95 per cent inactivation had been accomplished. Then more phage was added to the reaction vessel and its rate of inactivation followed. As the figure shows, the second quantity of phage was inactivated at the same rate as the first batch, indicating that the concentration of inactivator had not appreciably decreased.

Rate of Phage Inactivation at 37°C.

At this temperature phage inactivation followed the logarithmic curve for only about the first 50 per cent, after which the inactivation became slower. Furthermore, the relationship of initial rate to F concentration is different; the rate is nearly proportional to the concentration of F, not its

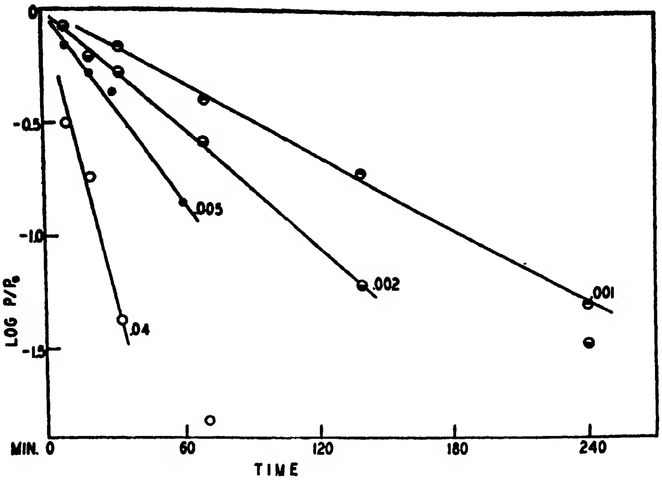

FIG. 1. The influence of F concentration on the rate of phage inactivation at 0°in broth. The numbers adjacent to the curves refer to the concentration of filtrate in the reaction mixture in arbitrary units.

TABLE I

The Influence of F Concentration on the Rate of Phage Inactivation at 0°C. in Broth

(F)	$\frac{-d \log (P)}{dt}$	k_0
0.04	0.042	0.21
0.005	0.015	0.21
0.002	0.009	0.20
0.001	0.006	0.19

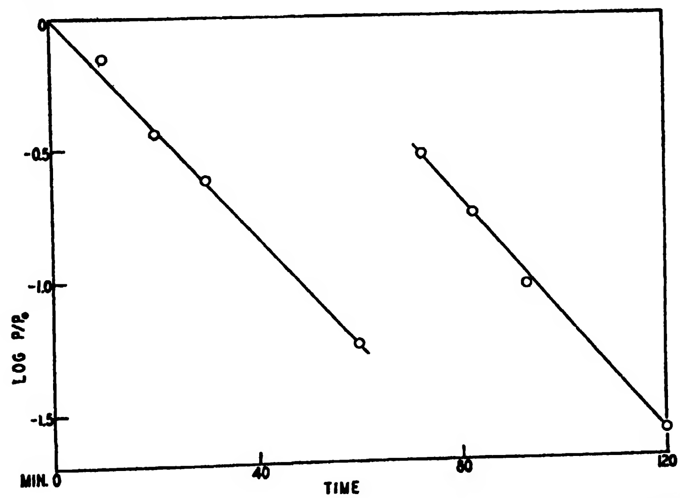

FIG. 2. The inactivation of a second portion of phage, after the inactivation of 95 per cent of a first portion, demonstrating the presence of excess inhibitor. The second portion of phage was added at 65 minutes.

square root. Fig. 3 shows the course of the inactivation reaction for three F concentrations, and Table II shows the corresponding values of the reaction rate constant, k_{37}, calculated from the equation

$$-\frac{d \log (P)}{dt} = k_{37}(F)$$

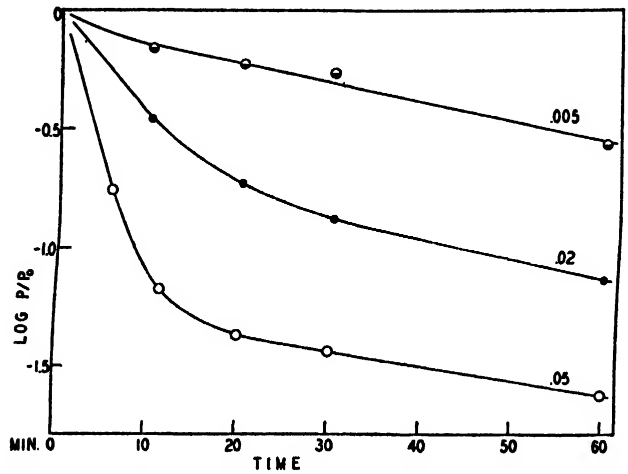

FIG. 3. The course of phage inactivation by filtrate at 37°C. in broth. The numbers adjacent to the curves refer to the concentration of filtrate in the reaction mixture in arbitrary units.

TABLE II

The Influence of F Concentration on the Rate of Phage Inactivation at 37°C. in Broth

(F)	$-\frac{d \log (P)}{dt}$	k_{37}
0.05	0.15	3.0
0.02	0.05	2.5
0.005	0.016	3.2

Clearly, the rates are here proportional to the concentration of F, not $F^{\frac{1}{2}}$ as at 0°C. Similar experiments at intermediate temperatures showed that the initial rates were proportional to (F) at 20°C., but became proportional to $(F)^{0.76}$ at 6°C.

Effect of Salt on the Inactivation

The inhibitor substance does not pass through a dialysis membrane and is probably a large molecule. Surface forces can be expected to play an important rôle in these reactions. These surface forces can be readily changed by changes in electrolyte concentration. The course of the phage

inactivation in the absence of significant amounts of electrolytes was investigated, by diluting both phage and inactivator in distilled water instead of broth. The effects of the addition of various concentrations of sodium chloride were also studied.

The addition to the broth used as the diluting medium of 25 per cent NaCl stopped the inactivation reaction. No significant decrease in phage occurred in 2 hours incubation at 37°C. in a filtrate concentration of 0.1. Without salt, at this (*F*) concentration, the phage would have been 90 per cent inactivated in less than 5 minutes. Similarly when tested at 0° this concentration of salt practically stopped the inactivation. On the other hand, salt concentrations of 0.5 per cent were found to increase the rate of inactivation. A 2.5 per cent concentration slightly decreased the rate of inactivation. In distilled water dilutions, where the only electrolytes were the impurities in the filtrate and phage, the inactivation did not proceed at an appreciable rate. Incubation of the phage without (*F*) under the same conditions of temperature and salt concentration resulted in no change in the amount of phage. This is contrary to a statement by Gratia (8) who attributed an inactivation of phage in a lysate with added salt to a direct action of the salt on phage. We believe that this effect of moderate salt concentrations in his case may have been not on phage directly but due to salt accelerated inactivation of phage by substances from the lysed bacteria.

The effects of salt concentration led us to test whether phage which had been inactivated by *F* could be regenerated by agents known to be effective dissociating agents. High concentrations of various salts, or low concentrations of soaps, were added to solutions in which phage had been inactivated with *F*, but without the slightest indication of the reactivation of any of the inactivated phage. These reagents alone had no effect on active phage, in any concentrations used.

Phage Inactivation by Common Polysaccharides

The inactivation of phage by several common polysaccharides was recently reported by Ashenburg *et al.* (5). We have confirmed their results with gum arabic and starch, with our phage, and have found in addition that inulin and acetylated gum arabic also will inactivate our *coli*-phage. The concentrations of starch or gum arabic needed to attain reasonable rates of inactivation (50 to 100 mg. per cc.) are greatly in excess of those required in the case of the specific substance from the bacteria. The preparations of inhibitor substance (*F*) used by us contained 2.5 mg. total solids per cc. Additional chemical work on this substance, still in progress,

shows that considerably more than half of this must be considered impurity. Therefore, it may be safely concluded that concentrations of inhibitor of less than 0.002 mg. per cc. produce a rapid inactivation of phage (Fig. 1).

At 0°C., the inactivation with starch is too slow to be measured. The course of the reaction at 37° with starch is similar to that with the bacterial inactivator. The decrease in phage is logarithmic at first, becoming slower in the later stages. This rate is proportional to the starch concentration. This is especially interesting in view of the high specificity of culture filtrates as inactivating agents, a specificity which parallels the adsorption of phage by the corresponding bacteria (1).

DISCUSSION

The interpretation of these data in terms of a reaction mechanism is complicated by several factors. The absolute concentration of inhibitor substance is unknown, and the relative molecular concentrations of phage and inactivator cannot be determined. Furthermore, this phage has not been isolated in high enough concentrations to make it feasible to study the course of the reaction in the presence of excess phage instead of excess inactivator. The change with temperature in the dependence of the rate on F concentration makes the consideration of temperature coefficients difficult. For example, at very low F concentrations, the inactivation at 0° is faster than that at 37° for the same concentration of inactivator.

We shall consider first only the initial rates, reserving for later discussion the slower rates attained after inactivation of the main fraction of phage. The assumption of the following series of reactions accounts for the observations in a simple way:

$$b + b \rightleftharpoons bb \qquad (1)$$

$$P + b \rightleftharpoons P{:}b \qquad (2)$$

$$P{:}b \rightarrow X \qquad (3)$$

The symbol $P{:}b$ represents a complex of phage and inactivator in which the phage retains temporarily full activity, while X represents the inactivated complex. "b" is the molecular species of the inactivator substance which combines with phage and "bb" is a dimolecular form which is inert toward phage. Reactions (1) and (2) are equilibria, and are rapid and reversible, and reaction (3) is the rate controlling irreversible step leading to inactivation of phage. Equilibrium (1), from our data, must be temperature sensitive, being pushed to the left by an increase in temperature. According to these reactions, phage is monovalent with respect to "b" in this inactivation process.

Taking these reactions as descriptive of the mechanism and with the assumptions stipulated above, a rate expression can be derived. The equilibrium constants for reactions 1 and 2 are respectively

$$K_1 = \frac{(bb)}{(b)^2} \tag{4}$$

$$K_2 = \frac{(P \cdot b)}{(P)(b)} \tag{5}$$

The total concentration (all forms) of inactivator is

$$(F) = (b) + 2(bb) \tag{6}$$

The rate of phage inactivation is

$$-\frac{d(P)}{dt} = k'(P \cdot b) \tag{7}$$

which by substitution from (5) becomes

$$-\frac{d(P)}{dt} = k'K_2(P)(b) \tag{8}$$

Substituting for (b) from (4) and (6) gives

$$-\frac{d(P)}{dt} = \frac{k'K_2}{4K_1}(P)\left(-1 \pm \sqrt{1 + 8K_1(F)}\right)$$

When the inactivator is chiefly in the "bb" form, K_1 is large and the rate expression becomes

$$-\frac{d(P)}{dt} = k_0(P)(F)^{\frac{1}{2}} \text{ where } k_0 = \frac{k'K_2\sqrt{2}}{2K_1^{\frac{1}{2}}}$$

which is the observed relationship at 0°C. When the inactivator is largely in the dissociated "b" form, the rate expression is substantially equation (8)

$$-\frac{d(P)}{dt} = k_{37}(P)(F) \text{ where } k_{37} = k'K_2$$

which is the observed relation at 37°C.

Although the equations assumed above lead in this way to rate expressions which agree with the experimental findings, this does not prove their reality. However, they permit discussion of the observations on definite terms.

The influence of salt on the course of the reaction may be described as an influence on reaction (2) in which a minimal electrolyte concentration is necessary to permit the combination of P and b, and high concentration

dissociates the $P \cdot b$ complex, preventing the inactivation by reaction (3). The precipitin reaction provides an analogy. Here, the presence of moderate amounts of electrolytes is necessary for the combination of antigen and antibody to proceed, whereas in strong electrolyte solutions, the combination does not occur (9).

After a part of the phage has been inactivated the rate decreased (see Fig. 3), indicating that a fraction of the phage particles was more slowly inactivated than the remainder. Schlesinger (10), and Delbrück (11) reported that in the adsorption on live and dead bacteria, there is a fraction of the phage which is less reactive than the remainder. This variation in adsorption rate of different fractions of the phage cannot explain the results obtained in the filtrate inactivation. At 0°C. the phage inactivation was first order until after inactivation of more than 95 per cent of the phage (see Fig. 1) while at 37°C. the rate was first order during the inactivation of about 90 per cent when $(F) = 0.05$, and about 65 per cent when $(F) = 0.02$ (see Fig. 3). Similar results were obtained in 2.5 per cent saline solutions.

From these results, and those previously appearing in the literature (3 and 4) it appears that there are two competing processes, one inactivating phage (the logarithmic part of the inactivation curve) and the other producing "partly inactivated phage." The second process, if more temperature sensitive than the first, would result in logarithmic inactivation over a greater range at 0° than at 37°C. The inactivation of the "partially inactivated phage" might occur by (*a*) reversal of the second process to permit inactivation by the first mechanism, or (*b*) a direct inactivation of the partially inactivated phage.

These considerations lead to the view that phage inactivation by filtrates is more than simply combination of phage with inhibitor substance, and that the extent of phage inactivation is not a direct measure of the extent of this combination. This view is supported by the observation of Andrewes and Elford (3) that phage ceases to pass through a membrane filter immediately after mixing with antiserum, indicating in this case the formation of a phage-antibody complex still retaining phage activity. In this regard, phage is similar to catalase (12) and urease (13), where the combination of the enzyme and anti-enzyme does not result in complete loss of activity.

SUMMARY

1. The rate of inactivation of an anti-*coli* phage by filtrates of cultures of the homologous bacteria has been studied.

2. The inactivation rate at 37°C. is proportional to phage concentration and filtrate concentration.

3. At 0°C. the rate of phage inactivation becomes proportional to the square root of the filtrate concentration.

4. A reaction scheme to account for these observations is suggested and discussed.

5. This *coli*-phage is also inactivated by relatively large concentrations of soluble starch, inulin, gum arabic, and acetylated gum arabic.

6. The inactivation is markedly influenced by salt concentration, being rapid at moderate salt concentrations and slow at high or extremely low salt concentrations.

7. The inactivated phage cannot be regenerated by high salt concentrations, or by soaps.

One of us (E. L. E.) wishes to acknowledge a grant in aid from Mrs. Seeley W. Mudd.

REFERENCES

1. Burnet, F. M., *J. Path. and Bact.*, 1934, **38,** 285.
2. Freeman, M., *Australian J. Exp. Biol. and Med. Sc.*, 1937, **15,** 221.
3. Andrewes, C. H., and Elford, W. J., *Brit. J. Exp. Path.*, 1933, **14,** 367, 376.
4. Burnet, F. M., Keogh, E. V., and Lush, D., *Australian J. Exp. Biol. and Med. Sc.*, 1937, **15,** 227.
5. Ashenburg, N. J., Sandholzer, L. A., Scherp, H. W., and Berry, G. P., *J. Bact.*, 1940, **39,** 71.
6. Ellis, E. L., and Delbrück, M., *J. Gen. Physiol.*, 1939, **22,** 365.
7. Delbrück, M., *J. Gen. Physiol.*, 1940, **23,** 643.
8. Gratia, A., *Compt. rend. Soc. biol.*, 1939, **132,** 62.
9. Heidelberger, M., Kendall, F. E., and Teorell, T., *J. Exp. Med.*, 1936, **63,** 819.
10. Schlesinger, M., *Z. Hyg. u. Infectionskrankh.*, 1932, **114,** 136.
11. Delbrück, M., *J. Gen. Physiol.*, 1940, **23,** 631.
12. Campbell, D. H., and Fourt, L., *J. Biol. Chem.*, 1939, **129,** 385.
13. Kirk, J. S., and Sumner, J. B., *J. Biol. Chem.*, 1931, **94,** 21.

ELECTROPHORETIC STUDIES ON HUMAN RED BLOOD CELLS

By ROBERT F. FURCHGOTT AND ERIC PONDER

(*From The Biological Laboratory, Cold Spring Harbor, Long Island*)

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These experiments were made to obtain more information about the surface of the red blood cell. Since "surface" means different things to different people, we shall define our meaning now. Strictly speaking, the surface studied by electrophoresis is the surface of shear between the cell (moving in the electric field) and the surrounding medium, for it is the potential at this surface which is the electrokinetic potential. But since this potential has its origin in the charged groups possessed by the cell membrane, we shall broadly use the term surface (unless we say otherwise) to mean that part of the membrane possessing these charged groups. That part of the membrane may be limited to the outermost constituent molecules of the membrane (*i.e.*, to those molecules just inside the surface of shear), but we have no direct evidence of this. Also we have no data on the variations of charge density in different portions of the surface.

The electrophoresis studies in this paper are divided into three parts. First, we have determined the mobility of human red cells as a function of the ionic strength at approximately constant pH. Secondly, we have determined the mobility as a function of pH at constant ionic strength for intact red cells, for the lipid of the red cell stroma, and for the protein of the stroma. Finally, we have determined the mobility of cells and ghosts under experimental conditions which cause changes in the mobility.

Methods and Preparations

1. Method of Electrophoresis.—The mobility measurements were carried out in an Abramson horizontal microelectrophoresis cell, using the technique of Abramson (1929, 1934) and of Moyer (1936). The cell was modified in two respects. The horizontal observation chamber dipped slightly below the level of the stopcocks and glass supporting rods, so that it rested on the microscope stage just above the condenser. This made it possible to use the cell with dark-field illumination (paraboloid) as well as with direct light. With direct light a Zeiss 28x ocular and 40x water-immersion objective were used, while with dark-field illumination we used the same ocular and a 20x high-dry objective. In the mobility measurements direct illumination was used except when specifically stated.

The other modification of the cell was the shortening of the vertical outlet tube over

one of the three-way stopcocks so that its top was a few centimeters below the top of the funnel-shaped inlet tube over the other three-way stopcock. We filled the cell rapidly while it was on the microscope stage in position for measurements. The cell, previously filled with the same solution as used for suspending the erythrocytes, was placed on the stage with the stopcocks adjusted to connect the inlet and outlet tubes by way of the observation chamber, and then the suspension of erythrocytes was poured into the inlet tube. This caused the solution originally in the cell to overflow out of the shortened outlet tube, while the suspension of erythrocytes in turn filled the cell. By pouring in a suspension immediately after making it, and by having electrical and optical adjustments for measurements approximately made before, we could make mobility measurements on the erythrocytes within 30 seconds of suspending them, which proved useful as we were often confronted with changes in mobility with time.

The mobility measurements were made at the "stationary levels." The specific resistance and the pH of each suspension were determined. The mobility measurements were made at room temperature, but all the mobilities were corrected to 25°C. Before each series of measurements the electrophoresis cell was cleaned with a concentrated Na_3PO_4 solution, and then, to ensure a uniform electroosmotic flow along the inside walls, a solution of serum was allowed to stand in the cell long enough for the walls to take on a coating of adsorbed protein.

2. Solutions.—What may be considered the standard reference solution for these experiments was an approximately isotonic mixture of nine parts of 1 per cent NaCl, 0.2 parts of M/15 KH_2PO_4, and 0.8 parts of M/15 Na_2HPO_4. This solution has an ionic strength of 0.172, a pH of 7.32 $\pm$ 0.2, and a specific resistance of 59.5 $\pm$ 0.3 ohms at 25°C. This will be called the "standard saline-phosphate solution."

For determining the effect of ionic strength on mobility, a series of mixtures of this standard solution and a 5.4 per cent glucose solution was made, containing per 100 parts respectively 50, 25, 10, 5, 4, and 2.5 parts of the standard solution. The pH values of the suspensions made with these mixtures were all over 7.0, except in the case of the last, where the pH was 6.85.

For the determination of the effect of pH on mobility at constant ionic strength, a series of mixtures of NaCl solution and various buffer solutions was made with the same ionic strength (0.172) as the standard saline-phosphate solution. The buffer solutions generally contributed about 0.1 of the total volume of the mixtures. The buffer systems were: M/10 NaOH—glycine—NaCl; M/15 Na_2HPO_4—KH_2PO_4; M/10 NaAC —HAc; M/10 HCl—glycine; and 0.13 M HCl.[1]

3. Preparation of Cells and Cell Products.—We obtained our cells from human blood drawn within a few hours of the electrophoresis measurements. It was mixed with about 100 times its volume of 1 per cent saline. The cells were centrifuged down, resuspended with 1 per cent NaCl in a conical centrifuge tube, and again centrifuged. The supernatant fluid was pipetted off. In making a suspension, enough cells were transferred on a stirring rod from the bottom of the centrifuge tube to the suspending solution so as to make a concentration of about 1 in 2000.

The lipid of the stroma was obtained by extracting intact cells at room temperature with a 3 to 1 mixture of ethyl alcohol (95 per cent) to diethyl ether, as was successfully

[1] All systems to be found in Clark's The determination of hydrogen ions, Baltimore, The Williams & Wilkins Co., 3rd edition, 1928.

used by Boyd (1936) and by Dziemian (1939). Intact cells were used, for it has been shown (Beumer and Bürger, 1912; Erickson *et al.*, 1938) that practically all the lipid of the red cell is contained in the stroma. The cells were obtained from freshly drawn, defibrinated human blood, and after being washed and packed, a few cubic centimeters were shaken with 30 volumes of the alcohol-ether solution for an hour. The mixture was then centrifuged, and the clear supernatant solution decanted into a suction flask. From the flask the solvent was evaporated under reduced pressure between 40 and 45°C., so that the extracted lipid was left as a thin skin on the bottom of the flask. By shaking the desired suspending solution in the flask and using a stirring rod to help to dislodge the lipid from the bottom, an emulsion of the lipid suitable for electrophoresis measurements can easily be made.

In obtaining the protein of the red cell stroma, washed ghosts were first prepared. Following the method of Parpart (1940), about 10 cc. of defibrinated and freshly drawn human blood were hemolyzed with 2 volumes of distilled water, and then about 20

TABLE I

Electrophoretic Mobility of Human Red Cells at 25°C. in Isotonic Mixtures (Salt and Glucose Solutions) of Various Ionic Strengths and Approximately Constant pH

Ionic Strength	$1/\kappa \times 10^8$	V μ/sec./volt/cm.	V, corrected for viscosity
0.172	7.37	−1.03	−1.04
0.086	10.40	−1.24	−1.35
0.043	14.71	−1.72	−1.92
0.017	23.12	−2.44	−2.78
0.0086	32.95	−2.80	−3.21
0.0069	36.80	−3.00	−3.44
0.0043	46.50	−3.16	−3.63

volumes of CO_2-saturated water at about 0°C. were added to the mixture. The flocculated ghosts were centrifuged down, and washed six times at room temperature with a 0.05 per cent NaCl solution. The supernatant fluid from the last washing, completed within an hour of the original hemolysis, appeared to be free of hemoglobin, although the ghosts, being of a pale pink color, contained a little of it. In order to extract the lipid from the protein of the ghosts, the packed, washed ghosts were shaken with 30 volumes of a 3 to 1 alcohol-ether mixture at room temperature for about 1 hour. The residue from the extraction (the protein of the stroma) was then separated from the extracting solution by centrifugation, washed a few times with an alcohol-ether mixture, and dried in a current of air. For the purpose of electrophoresis measurements, small portions were shaken vigorously with the desired suspending solution, so that we obtained small fragments suitable for the making of measurements.

RESULTS

1. *Mobility as a Function of Ionic Strength*

In Table I are shown the mobilities of human red cells in mixtures of various ionic strengths and approximately constant pH (7.3–7.0 for all

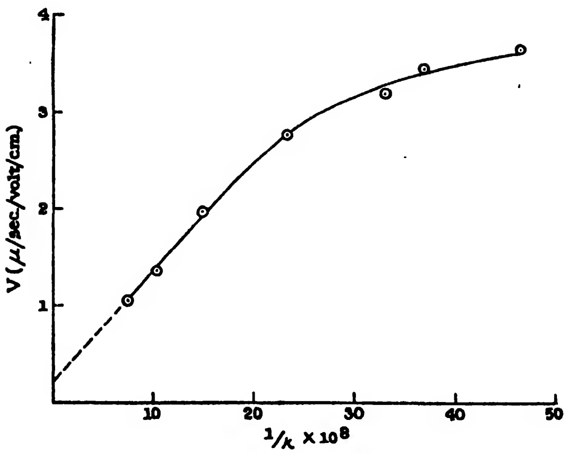

FIG. 1. Corrected mobility of human red cells as a function of $1/\kappa$.

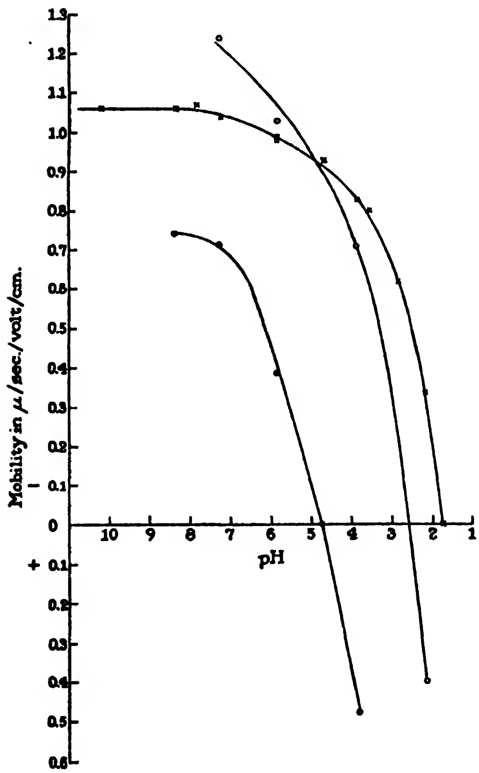

FIG. 2. Mobility as a function of pH for human red cells (crosses), for the lipid extract of the cells (open circles), and for the stroma protein of the cells (closed circles). This is at 25°C. and at ionic strength of 0.172.

except the mixture with an ionic strength of 0.0043 and pH of 6.85). In the second column of the table we have given the value of $1/\kappa$ for each mixture, where κ is the Debye function of ionic strength. (At 25°C., κ is 0.328×10^8 of the square root of the ionic strength.) In the fourth column of Table I we have given the mobilities corrected for viscosity, obtained by multiplying the observed mobilities of column 3 by the relative viscosities of the respective solutions used. In Fig. 1 are plotted the data of the second and fourth columns in Table I.

2. *Mobility As a Function of pH*

In determining the mobilities of human red cells at various pH levels and constant ionic strength, a phenomenon first emphasized by Abramson

TABLE II

Electrophoretic Mobility of Human Red Blood Cells at Various pH Levels and Constant Ionic Strength of 0.172 at 25°C.

pH	Buffer system used with NaCl solution to adjust pH	Mobility in μ/sec./volt/cm.
10.29	M/10 NaOH—Glycine—NaCl	−1.06
8.35	" " " "	−1.06
7.88	M/15 Na_2HPO_4—KH_2PO_4	1.07
7.32	" " " "	−1.04
5.88	" " " "	−0.98
5.87	M/10 HAc—NaAc	−0.99
4.70	" " "	−0.93
3.86	" " "	−0.83
3.53	M/10 HCl—Glycine—NaCl	−0.80
2.90	" " " "	−0.62
2.22	" " " "	−0.34
1.73	0.13 M HCl	0.0

(1930) has to be considered. This phenomenon is the variation of the mobility with time in suspensions of low pH (generally under pH 4). However, after numerous observations, using the technique (see Methods) which allows for the making of the first mobility measurements within 30 seconds, we found that the mobility remains substantially constant for about 3 minutes from the time of the first observation (except when lysis by acid occurred within this short time, in which case the mobility remained constant until the lysis: the mobility would then begin to vary in a manner which will be discussed in the next section). Therefore, in obtaining mobilities of red cells at any pH below pH 4, we made observations on a few suspensions at the desired pH, and then used only the mobility measurements made during the periods before the onset of the variations with time so as to obtain an average mobility for the particular pH.

Table II shows the mobilities of red cells at various pH levels at an ionic

strength of 0.172. Dark-field illumination was used in determining the mobilities at the two lowest pH levels in order that the mobilities of the ghosts formed by acid hemolysis could be observed also.

In Fig. 2 are plotted the data given in Table II, and also the pH-mobility points obtained for the lipid extract of the red cell stroma and for the lipid extracted protein residue of the red cell stroma. The mobility measurements on the lipid and protein (made with dark-field illumination) were not so satisfactory as those on the intact red cells. The coefficient of variation of a series of individual measurements (of time) on a single suspension in the case of these cell constituents was generally about $\pm$ 10 per cent, as compared with a coefficient of variation of about ± 5 per cent for a similar series of measurements on intact cells.[2] In fact, the point approximately at the isoelectric point on the protein curve is the average of measurements not only on particles with no mobility, but also on particles with very small positive mobilities and very small negative mobilities, whereas the point at the isoelectric point on the curve for intact cells is at the pH where all the cells were apparently stationary in the electric field.

3. *Variations in Mobility under Certain Conditions*

(*a*) The variation of mobility of red cells with time was found at all pH levels listed in Table II below pH 4.7. At pH 4.7, or at any higher pH levels, no variation was found during observations on a single suspension (taking usually about 15 minutes). At pH 3.8, where the variation was first noticed, the negative mobility of the cells began to decrease about 3 minutes after the mixing of the cell suspension, and in 10 minutes had fallen to half of the original value. At that pH, as well as at pH 3.5 and pH 2.9, the decrease in mobility began to occur before hemolysis occurred. At pH 2.2, however, hemolysis occurred about 3 minutes after the mixing, and the first change in the mobility began simultaneously with the hemolysis. This change in mobility was not a comparatively gradual decrease as was found at higher pH levels. The ghosts formed by the hemolysis from cells moving with a negative mobility began moving almost at once with a positive mobility. Likewise at pH 1.7, where the intact cells were stationary in the electric field, the ghosts formed by hemolysis (which was complete at this low pH within a minute) began to move at once with a positive mobility. The average positive mobility of the ghosts formed at this

[2] This coefficient of variation for measurements on intact cells is to be attributed more to errors in timing than to variations in mobilities. The individual mobility measurements were generally only about 7 seconds in length, and the stop-watch used recorded only 0.2 second intervals.

lowest pH was 1.0 μ/sec./volt/cm., and did not appear to change during several minutes following hemolysis. (Similar mobility reversals on hemolysis in acid solutions had formerly been observed by Abramson (1930) with sheep cells.)

(*b*) A decrease of the negative mobility of intact red cells with time also occurs when the suspending solution is a mixture of 97.5 parts of 5.4 per cent glucose and 2.5 parts of the standard saline-phosphate solution. This decrease was decidedly slower than any caused by low pH. It involved a fall from −3.16 to −2.93 μ/sec./volt/cm. in 1 hour, and to −1.86 μ/sec./volt/cm. in 2 hours,[3]

(*c*) Finally, one other change of mobility should be mentioned. This change was a decrease in the mobility of ghosts prepared by Parpart's method (1940) (see Methods). It has been previously shown by us (Abramson, Furchgott, and Ponder, 1939) that unhemolyzed rabbit red cells and ghosts made by various forms of lysis without subsequent CO_2-flocculation have the same mobility in a solution of glucose and phosphate buffer. Here we found that the ghosts of human red cells made by hypotonic lysis without subsequent CO_2-flocculation have the same mobility (−1.04 μ/sec./volt/cm.) as unhemolyzed human red cells in the standard saline-

[3] In the case of the measurements at low ionic strengths (10 parts or less of standard saline-phosphate solution in 100 parts of the suspending mixture), it was found that small traces of $CuSO_4$ (of the order of 0.001 per cent in the case of a suspension containing 5 parts of the standard saline-phosphate solution per 100 parts) were capable of markedly and rapidly decreasing the red cell mobility, sometimes even to the extent of reversing the sign of the mobility. The pH decreases caused by such traces of copper salt were only about 0.1 of a pH unit and therefore of no consequence. The mobility decreases of this sort were first encountered when, because of inadequate washing of the electrophoresis cell before filling it with an erythrocyte suspension, traces of $CuSO_4$ from the electrode plugs contaminated the suspension. Somewhat similar mobility decreases in the presence of Cu^{++} and certain other metal ions had been previously observed by Northrop and Freund (1923) and by Oliver and Barnard (1924).

Traces of $CuSO_4$, however, did not change the mobilities of the cells in the mixtures of standard saline-phosphate solution and 5.4 per cent glucose solution of higher ionic strengths (*e.g.*, in a 1:1 mixture of these solutions). This was also the case when a solution of unbuffered 1 per cent saline was used as the suspending medium. Apparently the surfaces of the cells in the solutions of high glucose and low salt content were somehow changed from what they were in solutions of high salt content, so that they were capable of adsorbing cupric ions, thus changing their electrokinetic potential. Also there was no mobility change with traces of $CuSO_4$ in a mixture of 90 parts of glucose solution and 10 parts of M/15 phosphate buffer (pH 7.38). In this case the cupric ions, despite the relatively low total salt content, probably are prevented from being adsorbed on cells in detectable quantities because of the formation of poorly ionized complexes between them and the relatively abundant phosphate and acid phosphate ions.

phosphate mixture, whereas ghosts subjected to the CO_2-flocculation and washing of Parpart's method have a mobility of only -0.85 μ/sec./volt/cm. in the same mixture. Ghosts prepared by Parpart's method also behave differently from ghosts hemolyzed by hypotonicity which have been subjected to less drastic treatment in that they do not disintegrate into stromatolytic forms on treatment with solutions of lyotropic salts such as lithium perchlorate (Furchgott, 1940).

DISCUSSION

From the results of the mobility measurements at various ionic strengths we obtain information about the contour of the red cell surface. Let us consider the curve in Fig. 1 in the light of Gorin's recent equations (Abramson, Gorin, and Moyer, 1939). Gorin's general equation (his equation 2′) when applied to the limiting case of particles of very large radius of curvature, gives us

$$V = \sigma(1/\kappa + r_i) \tag{1}$$

where V is the mobility in μ/sec./volt/cm. corrected for the viscosity of the medium, σ is the charge density of the surface of the particle, κ is the Debye function of the ionic strength, and r_i is the mean of the radii of the ions in the diffuse double layer. This equation predicts that for particles of very large radius of curvature V is a linear function of $1/\kappa$ if the charge density remains constant.

However, for some particles which microscopically appear to have large enough radii to satisfy equation (1), measurements over ionic strength ranges in which the charge density varies inappreciably give non-linear V-$1/\kappa$ curves. From evidence obtained largely with microscopic particles coated with adsorbed protein, it appears that these non-linear curves are the result of "bumpy" surfaces, with the effective radius of curvature possibly being the radius of curvature of the individual bumps (Abramson, Gorin, and Moyer, 1939). Conversely it appears that for particles of large "gross" radius of curvature and constant charge density, deviation from linearity of the V-$1/\kappa$ curve may indicate a bumpy surface.

Looking back to Fig. 1 now, we see that below $1/\kappa$ of about 20×10^{-8} (equivalent to an ionic strength of about 0.02), V is a linear function of $1/\kappa$. Assuming that the charge density is almost constant over this range, our curve shows that the red cell surface behaves at ionic strengths above 0.02 as a smooth surface with a very large radius of curvature. Further evidence for the applicability of equation (1) to the present data is the value of r_i obtained by dividing the intercept of the curve by the linear slope. The

value is 1.8 Å, which is of the right order of magnitude for the mean of the radii of the ions (mostly Na^+ and Cl^-) in the diffuse double layer (Gorin, 1939).

At values of $1/\kappa$ above about 20×10^{-8} (μ values below about 0.02), the curve in Fig. 1 is no longer linear. This, in our opinion, indicates that changes in the surface of the cell occur in solutions of $\mu < 0.02$. A change from a smooth to a bumpy surface would decrease the slope of the curve, but it is more likely that the falling of the slope in Fig. 1 is a result of a decrease in charge density with the decrease of ionic strength. Such decreases of charge density in solutions of low ionic strength are a well known phenomenon with various kinds of surfaces (Abramson and Müller, 1933). Other observations lead us to believe that the falling off of the slope of the curve in solutions of high glucose and low salt concentration may actually be connected with injury to the cell surface. By this we mean a change in the kind of molecules or in the arrangement of molecules in the surface.

Turning now to the pH-mobility curves in Fig. 2, the interesting point is the low isoelectric point of the red cell surface. This isoelectric point and also other points on the same curve below pH 4.0 were obtained before the onset of changes in mobility encountered at low pH levels. These changes in mobility with time, which may be due to adsorption of proteins (possibly hemoglobin where hemolysis is occurring) on the cell surface at low pH levels, have led to the reporting of wrong isoelectric points for red cells. Abramson (1930) has previously discussed this matter in some detail.

White and Monaghan (1936) have reported isoelectric points for ghosts (made by a somewhat drastic method of preparation) and lipid-extracted ghosts of cow, dog, and rabbit cells, but they did not make their measurements at corrected (for the "lens effect" of the cylindrical cell which they used) stationary levels. The isoelectric point which we have obtained for the lipid-extracted ghosts of human red cells (*i.e.*, for the stroma protein) is (Fig. 2) at about pH 4.7. This is not an unusual isoelectric point for a protein, and is not in disagreement with the amino acid analyses which have been made on stroma protein (Jorpes, 1932; Beach *et al.*, 1939).

Not only the isoelectric points, but also the entire pH-mobility curves for intact human red cells and for the stroma protein from them are decidedly different. Obviously the red cell surface is not a surface of stroma protein. The curve for the extracted lipid of the red cells is somewhat closer to that for the intact cells, but here again there are definite differences. The isoelectric point (by interpolation) of about pH 2.6 for the lipid is almost one pH unit higher than the isoelectric point of the cells, and the

curve for the lipid rises considerably above the flat maximum level found for the cells at alkaline pH values. We can therefore say that the surface of the human red cell does not have the composition of the surface of emulsified particles of lipid extracted from the cell membrane.

Looking once more at Fig. 2, especially at the unusually low isoelectric point and the high flat maximum mobility level above pH 7 in the curve for intact cells, it seems to us that the surface of the human red cell is dominated by strongly acidic groups. In view of the large cephalin content of these cells (Erickson *et al.*, 1938; Dziemian, 1939), it may be that these groups are the phosphoric acid groups of cephalin molecules. A surface of oriented molecules of cephalin and other lipids would probably satisfy our electrokinetic data, but the data, admittedly, do not rule out the possibility of smaller amounts of protein being in the surface also.

SUMMARY

1. The electrophoretic mobility of unhemolyzed human red cells has been determined as a function of ionic strength at approximately constant pH in isotonic mixtures of glucose solution and saline-phosphate buffer solution.

2. Above an ionic strength of about 0.02 the cells behave as particles with a smooth surface of large radius of curvature. Below an ionic strength of about 0.02, changes of the surface occur, probably involving a decrease of charge density and perhaps connected with injury of the surface.

3. The mobility as a function of pH at an ionic strength of 0.172 has been determined for human red cells, for the lipid extract of the cells, and for the stroma protein of the cells. The isoelectric points of cells, lipid, and protein have been found to be about 1.7, 2.6, and 4.7 respectively.

4. The pH-mobility data lead to the conclusion that a red cell surface is composed largely of lipid and dominated by strong acid groups, possibly the phosphoric acid groups of cephalin molecules.

To Dr. M. H. Gorin, now of the Magnolia Petroleum Co., Dallas, Texas, and to Dr. H. A. Abramson of the College of Physicians and Surgeons, Columbia University, we extend our thanks for their interest and suggestions relative to the work in this paper.

REFERENCES

Abramson, H. A., *J. Gen. Physiol.*, 1929, **12**, 711.

Abramson, H. A., *J. Gen. Physiol.*, 1930, **14**, 163.

Abramson, H. A., Electrokinetic phenomena, New York, The Chemical Catalog Co., 1934.

Abramson, H. A., Furchgott, R. F., and Ponder, E., *J. Gen. Physiol.*, 1939, **22**, 545.

Abramson, H. A., Gorin, M. H., and Moyer, L. S., *Chem. Rev.*, 1939, **24**, 345.

Abramson, H. A., and Müller, H., Cold Spring Harbor symposia on quantitative biology, Cold Spring Harbor, Long Island Biological Association, 1933, **1**, 29.

Beach, E. F., Erickson, B. N., Bernstein, S. S., Nims, B., and Macy, I. G., *J. Biol. Chem.*, 1939, **128**, 339.

Beumer, H., and Bürger, M., *Arch. exp. Path. u. Pharmakol.*, 1912, **71**, 311.

Boyd, E. M., *J. Biol. Chem.*, 1936, **115**, 37.

Clark, W. M., The determination of hydrogen ions, Baltimore, The Williams & Wilkins Co., 3rd edition, 1928.

Dziemian, A. J., *J. Cell. and Comp. Physiol.*, 1939, **14**, 103.

Erickson, B. N., Williams, H. H., Bernstein, S. S., Arvin, I., Jones, R. L., and Macy, I. G., *J. Biol. Chem.*, 1938, **122**, 515.

Furchgott, R. F., Cold Spring Harbor symposia on quantitative biology, Cold Spring Harbor, Long Island Biological Association, 1940, **8**, 100.

Gorin, M. H., *J. Chem. Physics*, 1939, **7**, 405.

Jorpes, E., *Biochem. J.*, London, 1932, **26**, 1488.

Moyer, L. S., *J. Bact.*, 1936, **31**, 531.

Northrop, J. H., and Freund, J., *J. Gen. Physiol.*, 1923, **6**, 60.

Oliver, J., and Barnard, L., *J. Gen. Physiol.*, 1924, **7**, 99.

Parpart, A. K., and Dziemian, A. J., Cold Spring Harbor symposia on quantitative biology, Cold Spring Harbor, Long Island Biological Association, 1940, **8**, 17.

White, H. L., and Monaghan, B., *Am. J. Physiol.*, 1936, **115**, 31.

A NEW METHOD FOR THE STUDY OF DIFFUSION OF BIOLOGICALLY ACTIVE MATERIAL

By JAQUES BOURDILLON

(From the Laboratories of the International Health Division of The Rockefeller Foundation, New York)

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The optical study of diffusion, which has yielded such brilliant results in recent years, is suited only to substances which can be obtained in pure solution. It cannot be applied successfully to mixtures of several components, nor to suspensions of material such as animal viruses, the actual concentration of which, in terms of weight of substance per volume of solution, is in general unknown. For these the study of diffusion must be analytical, that is, consist in sampling and testing quantitatively for specific activity.

Unfortunately, analytical diffusion is beset with numerous technical difficulties which have not yet been completely eliminated. Convection currents due to temperature gradients, the effects of vibrations, and difficulties in sampling represent probably the most common sources of error. The ingenious solution given the problem by Northrop and Anson (1) has been thus far the most satisfactory. This paper describes a different and very simple analytical diffusion method applicable to biological substances. Though it is probably not entirely free from the hazards enumerated above, it has been found suitable for a study of crystalline beef liver catalase, the results of which are reported below, and has been applied since to an investigation of the diffusion of several viruses.

Theoretical

The mathematical solution of a problem of diffusion depends on the particular experimental conditions. The procedure employed here consisted of superimposing a layer of water or buffer on a solution containing the material under investigation, allowing the latter to diffuse upward, sampling at various levels, and determining the concentration in the samples. Under such experimental conditions, the general differential equation $dc/dt = Dd^2c/dx^2$ has the particular solution

$$1 - 2C_x/C_0 = \frac{2}{\sqrt{\pi}} \int_0^y e^{-y^2}\, dy \tag{1}$$

in which

$$y^2 = x^2/4Dt$$

x is the distance in centimeters measured vertically from the initial boundary; D is the diffusion constant; t is the time in seconds. In equation (1) C_x is the concentration x centimeters from the initial boundary; C_0 is the initial concentration of the solution when $t = 0$; and the right-hand member is the probability integral, the value of which can be found in tables.

If one assumes that the diffusing particles are spherical, the molecular weight of a substance can be calculated from the diffusing constant alone. For $D = RT/N \cdot 1/f$, in which R is the gas constant, T the absolute temperature, N the Avogadro number, and f the frictional resistance opposed by the surrounding medium. For spherical particles, $D_0 = RT/N \cdot 1/f_0$, so that $D_0/D = f/f_0$. The term f/f_0 expresses the ratio of the resistance offered a nonspherical particle to that offered a spherical particle of the same mass. For a spherical particle of colloidal dimensions, $f = f_0 = 6\pi\eta r$, in which η is the viscosity of the solution at temperature T, and r is the radius of the particle. The molecular weight of the substance is then $4/3 \cdot \pi r^3 \rho N$, in which ρ is the density of the particle. A very accurate value for the molecular weight thus calculated can, of course, hardly be expected since the value obtained for D becomes cubed in the calculation, and since f/f_0 remains unknown.

It is important to note from equation (1) that all that is needed for the calculation of D is the relative concentration C_x/C_0 of the solution at a given distance from the initial boundary after a given time, and that the actual concentration, in terms of weight of substance per volume of solution, is not required. Without this advantage the diffusion study of most viruses could not be attempted.

The accuracy of the value obtained for D from one single sample removed after diffusion will depend on the accuracy of the analytical method applied. We have found that the most satisfactory procedure consisted in determining the concentrations of as many samples as possible, plotting them on a chart against the vertical distance above the initial boundary, and determining the theoretical curve which gives the best fit. If the substance under investigation can be determined in very low concentration, the values of relative concentration obtained are plotted logarithmically as the ordinates, against the vertical distances in the cell as the abscissae. For $x = 0$, C_x/C_0 will always be equal to 0.5, no matter how long diffusion is allowed to proceed. This procedure was used in the present investigation with catalase, which served as a test substance, and will have to be applied in the study of viruses.

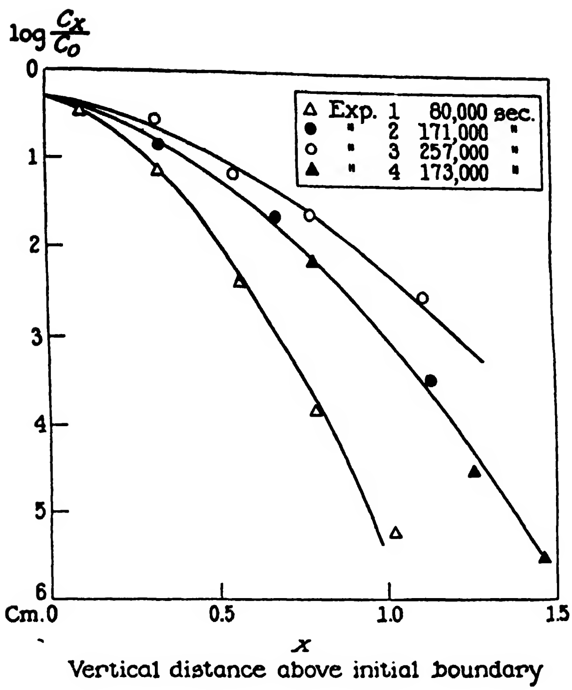

FIG. 1. Diffusion of catalase. Temperature 4°C. Theoretical curves calculated for $D = 3.1 \times 10^{-7}$ cm.^{2}/sec.

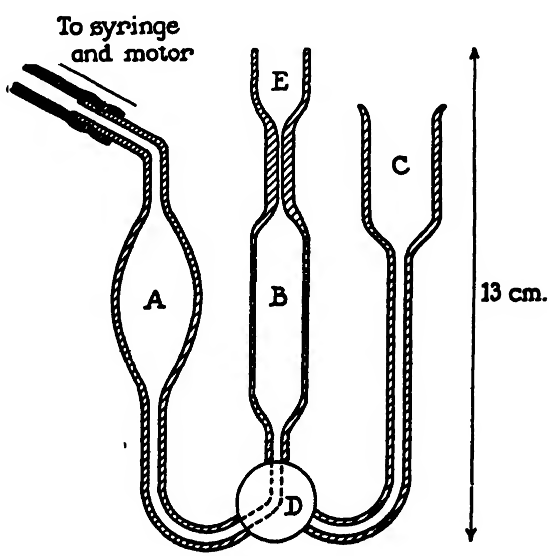

FIG. 2. Diagram of diffusion cell.

Now since the curves thus obtained (Fig. 1) become more vertical with increasing values of $-\log C_x/C_0$, the experimental error inherent in the concentration determination of each sample, though remaining of the same magnitude, becomes of less importance the smaller the relative concentration. On the other hand, since x appears to the square and D to the first power, experimental error in the computation of x will have an important effect on the value derived for D, and this error will be relatively greater the smaller the value of x. Consequently, the best experimental conditions will be realized when the material investigated can be determined in very low relative concentration and when diffusion is allowed to proceed long enough to make sampling possible at a sufficient height above the initial boundary.

Apparatus

Description.—The apparatus used is illustrated in Fig. 2. It is entirely made of glass and consists of two bulbs, A and B, and one large cup, C, all linked through glass tubing with the same stopcock, D. By turning the stopcock by 120°, A may be connected with B, B with C, or A with C. The bulb in the center, B, is the diffusion cell. It is exactly cylindrical on a length of about 3 cm., and its capacity is about 5 cc. Its upper end narrows to a capillary less than 1 mm. in inner diameter and about 1 cm. in length, which widens again into the small sampling cup, E. The capacity of C and A is about 6 cc. each. The upper end of A is connected through a section of rubber tubing with a 10 cc. glass syringe (not shown in diagram). The plunger of the syringe is operated by a synchronous electric motor.

Filling.—The apparatus is first entirely filled with water or buffer and immersed in a constant temperature water bath, the water completely covering cell B. Bulb A is connected with the rubber tubing and syringe, which are also filled with water. Cup C is emptied by moving the plunger back until the fluid in C just reaches the bottom of the cup; the last drops in C are mopped up with filter paper. The solution to be studied is then poured into C, the stopcock turned so as to connect C and A, and, by pulling the syringe plunger slowly by hand, the fluid is displaced from C to A until the meniscus in C reaches the bottom of the cup. The plunger is geared to the motor for forward motion, the stopcock is turned so as to connect A with B, and the motor is started and allowed to run until the boundary formed reaches about the middle of B, the duration of the process being exactly recorded. The stopcock is turned to connect A with C, and the apparatus is allowed to stand the necessary number of days. Fig. 2 shows that this arrangement permits the formation of a sharp boundary.

Sampling.—Cup E is emptied with a thin pipette, the stopcock turned to connect A with B, and the motor started; the fluid which comes out is pipetted from cup E at intervals while the time is exactly recorded.

In the apparatus used by the author the cross-section of the cell was 1.10 $cm.^2$; the motor displaced 39 $mm.^3$ of fluid per minute. From these data and from the time record of each experiment the mean height of each sample above the initial boundary was easily calculated. The apparatus was placed in the same vibrationless water bath used with the Tiselius electrophoresis cell. The temperature was about 4 ± 0.01°C.

Diffusion of Catalase

Material.—Four times recrystallized beef liver catalase, prepared by the method of Sumner and Dounce (2)[1] was used. The crystals, suspended in water, were dissolved with the help of solid NaCl and phosphate buffer to make a solution which was about M/1 in NaCl and M/10 in Na phosphate, with a pH of 7.4. The total salt concentration was thus about 7 per cent; the catalase concentration was 1 or 2 per cent. The solution was perfectly clear and would keep so for weeks; no residue was ever observed.

This material was found to be very nearly homogeneous by optical diffusion, with a diffusion constant of 4.4×10^{-7} at 20°C., and a "Kat. *f*."[2] of 35,500 (3). For previous preparations Sumner and Gralén found a molecular weight of 248,000, derived from a sedimentation constant of 11.2×10^{-13}, a diffusion constant of 4.1×10^{-7} at 20°C., and a partial specific volume of 0.73 (4). Calculation of the asymmetry factor gave $f/f_0 = 1.25$ (5). The isoelectric point was at pH 5.7 (6).

Determination of Catalase Concentration.—Catalase activity in the diffusion samples was determined as follows: Into a large test tube were introduced 5 cc. of 0.01 N H_2O_2, 1 cc. of 0.05 N Na phosphate buffer pH 6.8, and 0.1 cc. of an adequate dilution in water of the unknown diffusion sample. The test tube was kept at 0° and 1 cc. aliquots were removed from time to time. A drop of 25 per cent H_2SO_4 was immediately added to them, and they were titrated with 0.005 N $KMnO_4$. The reaction constant was obtained from the equation $k = 1/t \cdot \log \frac{a}{a - x}$, in which t is the time, a the original amount of peroxide, and $a - x$ the amount left after time t. Since the value of the reaction constant dropped slowly with time, the value chosen for k was that found by interpolation for $a/(a - x) = 2$. In some cases, in order to determine the smallest possible catalase concentrations, the reaction was allowed to proceed overnight; thus, amounts of catalase equal to about 0.003 micrograms were estimated with sufficient accuracy. The reaction constant being proportional under given conditions to the amount of catalase present, the relative catalase concentration in the diffusion samples could be directly obtained.

RESULTS

The results of four experiments are given in Fig. 1 on which the logarithms of the relative concentrations of catalase have been plotted against the

[1] We are much indebted to Dr. J. B. Sumner and Dr. A. L. Dounce for supplying us with a sample of their material.

[2] "Kat. *f*" is equal to k monomolecular per gram enzyme in 50 cc. reaction mixture, as defined by Euler and Josephson (Euler, H. von, and Josephson, K., *Ann. Chem.*, 1927, **452**, 158).

vertical distances measured from the initial boundary. In experiments 1 and 2 the original catalase solution was diffused against water for 80,000 and 171,000 seconds, respectively. In experiment 3 the catalase solution was first dialyzed against buffer (M/1 in NaCl and M/10 in Na phosphate, pH 7.4) and then allowed to diffuse into another batch of the same buffer for 257,000 seconds; in experiment 4 diffusion was allowed to take place into buffer for 173,000 seconds, but the latter had previously been slightly diluted with water so as to create a difference in salt concentration between the lower and the upper solution of about 1 gm. per 100 cc. Theoretical curves have been drawn on the chart to give the best fit for all experiments. (The curves for experiments 2 and 4 come so close to each other that only one has been drawn.) They all express the ideal diffusion of particles with a diffusion constant, D, equal to 3.1×10^{-7} at 4°C. From the degree of scattering of the points on the chart, this figure can be estimated to be correct within approximately plus or minus 5 per cent.

In experiments 1 and 2 the diffusion of catalase was accompanied by the simultaneous diffusion into water of a 7 per cent salt solution; in experiment 3 catalase alone was diffusing into buffer; in experiment 4, a 7 per cent salt solution was diffusing into a 6 per cent salt solution. These varying conditions did in no case influence the diffusion of catalase to an appreciable degree.

In one experiment only (not reported) appreciable mixing occurred. All samples above $x = 0.5$ yielded the same concentration as at $x = 0.5$; in that case catalase alone was diffusing (as in experiment 3). At $x = 0.5$ the concentration gradient of a protein under similar conditions is near zero. In this experiment it was probably insufficient to oppose accidental convection currents.

In a control experiment, in which catalase diffused into pure water, filling of the cell was followed immediately by sampling without stopping the motor. The total time was 7650 seconds. Between $x = 0$ and $x = 0.23$, the mean relative concentration found was -0.8 log; between $x = 0.23$ and $x = 0.45$, -3.1 log; no catalase was detected above. This is probably as good a result as can be expected when sampling is attempted immediately after filling and has to take place in a region where the concentration gradient is exceedingly steep.

The value of 4.4×10^{-7} obtained for the diffusion constant by optical measurements at 20°C. (3) becomes 2.7×10^{-7} at 4°C. after correction for temperature and viscosity. The value obtained by us of 3.1×10^{-7} is therefore in fair agreement, considering the nature of the method employed. Assuming the validity of such a correction even over a tempera-

ture difference of 16°, the slight discrepancy found might be explained by a systematic error in the calculation of the mean height of the samples, resulting in a shift of all points toward the right side of the chart, or by the simultaneous diffusion of a salt, as in experiments 1 and 2, or finally by such external causes as vibrations or temperature fluctuations. Any gross heterogeneity in the active diffusing material would have distorted the observed curves and yielded straight lines or curves concave toward the upper right corner of the chart, which is obviously not the case. Any small heterogeneity would have passed unnoticed. From what is known of the chemical constitution of catalase (2) one cannot expect the molecules to split into active fractions smaller than halves.

The results reported above show that this diffusion method can be used successfully for the study of biologically active material and that correct results can be obtained from samples withdrawn as high as 1.5 cm. above the initial boundary, whose concentration is less than one hundred thousandth that of the original solution. The simultaneous diffusion of a salt, which creates a concentration gradient moving ahead of the protein, does not affect appreciably the diffusion of the latter and seems to be a satisfactory way of avoiding possible erratic results due to convection. The technique is now being applied to the study of some viruses.

SUMMARY

A simple diffusion apparatus has been described in which a layer of solution is allowed to diffuse upward into a layer of solvent. Accurate sampling is performed at various heights and the concentration of the samples is determined.

The method has been illustrated with a determination of the diffusion constant of crystalline catalase, which was found to be 3.1×10^{-7} cm.^{2}/sec. at 4°C. The method should be especially suited to the study of biological substances endowed with specific activity and which cannot be obtained in pure solution.

REFERENCES

1. Northrop, J. H., and Anson, M. L., *J. Gen. Physiol.*, 1928–29, **12**, 543.
2. Sumner, J. B., and Dounce, A. L., *J. Biol. Chem.*, 1939 **127**, 439.
3. Sumner, J. B., and Dounce, A. L., personal communication.
4. Sumner, J. B., and Gralén, N., *J. Biol. Chem.*, 1938, **125**, 33.
5. Svedberg, T., and Pedersen, K. O., The ultracentrifuge, The Oxford University Press, 1940, 40, 406.
6. Sumner, J. B., and Dounce, A. L., *J. Biol. Chem.*, 1937, **121**, 417.

THE STRUCTURE OF THE COLLODION MEMBRANE AND ITS ELECTRICAL BEHAVIOR

I. The Behavior and Properties of Commercial Collodion

By KARL SOLLNER, IRVING ABRAMS, and CHARLES W. CARR

(From the Department of Physiology, University of Minnesota, Minneapolis)

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In the past there have been very many investigations of the behavior of collodion membranes with special attention to the phenomena and problems associated with electrolyte diffusion, or more properly, ion penetration. However, the fundamental characteristics of such membranes are still inadequately understood. Highly dried membranes with low permeability and high selectivity were investigated thoroughly by Michaelis and his co-workers.[1] Their work and that of later students has not, however, satisfactorily elucidated the more intimate reasons for the behavior of such membranes. The characteristics and behavior of membranes of greater permeability, which have even more biological significance, have received less attention than they deserve.

The absolute necessity of further investigation of the properties of collodion membranes has recently been emphasized by our indifferently successful attempts[2] to reproduce the fundamental observations of Loeb[3] on anomalous osmosis. Using a number of brands of collodion, only one (foreign) brand gave anomalous osmosis approaching the findings of Loeb.

This unexpected difference between different collodion preparations immediately suggested that even a partial clarification was bound to be of considerable importance. In order to be able to continue current investigations, we were forced to clarify this matter since, due to the war, it became

[1] Michaelis, L., and Fujita, A., *Biochem. Z.*, Berlin, 1925, **158**, 28; 1925, **161**, 47; 1925, **164**, 23; Michaelis, L., and Dokan, Sh., *Biochem. Z.*, Berlin, 1925, **162**, 258; Michaelis, L., and Hayashi, K., *Biochem. Z.*, Berlin, 1926, **173**, 411; Michaelis, L., and Perlzweig, W. A., *J. Gen. Physiol.*, 1927, **10**, 575; Michaelis, L., McEllsworth, R., and Weech, A. A., *J. Gen. Physiol.*, 1927, **10**, 671; Michaelis, L., Weech, A. A., and Yamatori, A., *J. Gen. Physiol.*, 1927, **10**, 685; Michaelis, L., *Bull. Nat. Research Council, No. 69*, 1929, 119; *Kolloid-Z.*, 1933, **62**, 2; and other publications.

[2] Sollner, K., and Abrams, I., *J. Gen. Physiol.*, 1940, **24**, 1.

[3] Loeb, J., *J. Gen. Physiol.*, 1918–19, **1**, 717; 1919–20, **2**, 173, 225, 387, 563, 577, 659, 673; and many other papers in the succeeding volumes of the same *Journal*.

more and more difficult, finally impossible to procure the (imported) collodion which had the desired properties.

The resulting investigation has led to an increased knowledge of the factors responsible for the electrochemical behavior of collodion membranes. Furthermore, we are now able to prepare membranes of a very high degree of electrochemical activity at will in the laboratory (Part II, to be published later).

A review of the literature showed that other investigators have had similar experiences when studying the electrical, particularly electromotive properties of collodion membranes.

In 1927 Michaelis and Perlzweig[4] tested at least ten different nitrocellulose preparations before finding one suitable for their studies of the electromotive behavior of highly dried collodion membranes. Only "Celloidin Schering" gave membranes which consistently showed maximum concentration potentials and reasonably measurable permeabilities (electric conductivity). The other preparations yielded membranes showing very poor electrical conductivity (permeability), low concentration potentials, or a combination of the two.

In 1935, Wilbrandt[5] (in Michaelis' laboratory) observed the same fact again and remarks that "Kollodium Schering-Kahlbaum DAB 6" "yielded the membranes with the highest and the most consistent concentration potentials, while with other types of collodion lower and less consistent potentials were obtained . . . . " ". . . .the membranes with low potentials often had a high resistance and vice versa." Table I shows the potentials of various types of membranes as found by Wilbrandt.

Some results concerning anomalous osmosis which have been obtained consistently by the present authors are summarized in Table II. It should be stated that anomalous osmosis is necessarily a very sensitive indicator of the electrical activity of a membrane.[6] The values given are pressure rises in a manometer tubing after 20 minutes when a bag containing the solution indicated is placed into distilled water. For details of the technique the reader is referred to our previous paper. The membranes used

[4] Michaelis, L., and Perlzweig, W. A., *J. Gen. Physiol.*, 1927, **10**, 575.

[5] Wilbrandt, W., *J. Gen. Physiol.*, 1935, **18**, 933.

[6] According to Loeb's experiments (see footnote 3) and the theory of one of us (Sollner, K., *Z. Elektrochem.*, 1930, **36**, 36, 234), the extent of anomalous osmosis is proportional to the product of electrokinetic (ζ) potential and membrane (ϵ) potential. As both of these magnitudes are directly dependent upon the electrochemical structure of a membrane, it is easily understood that any phenomenon which is proportional to the product of the two must necessarily be a very sensitive indicator of the "activity" of a membrane.

were, as far as possible, of the same porosity, as tested by their behavior towards sugar solution. No amount of effort and experimenting (*e.g.*, changing the porosity) obliterates these characteristic differences between

TABLE I

(*After Wilbrandt*)

Concentration Potentials of Collodion Membranes of Various Brands of Collodion, Measured between N/100 and N/1000 KCl (in mv.)

Brand of collodion	Concentration potential	
	Highest value	Lowest value
Collodion Mallinckrodt	25	16
Collodion Merck U.S.P. X	48	10
Kollodium Schering-Kahlbaum "zur Herstellung von Membranen"	50	38
Kollodium Schering-Kahlbaum "zur Analyse"	46	35
Kollodium Schering-Kahlbaum DAB 6	56	50

TABLE II

Anomalous Osmosis through Membranes Prepared from Several Brands of Collodion

1	2	3	4	5
Brand of collodion	Osmotic rise with sugar $\frac{M}{4}$	Anomalous osmosis		
		KCl $\frac{M^*}{256}$	K_2SO_4 $\frac{M^*}{512}$	K_3-citrate $\frac{M^*}{128}$
		mm.	mm.	mm.
"Parlodion" Mallinckrodt	130	8	35	24
Collodion Merck U.S.P.	128	14	54	112
Collodion Baker U.S.P.	128	6	48	130
Collodium Schering-Kahlbaum "pro analysi"	122	8	55	132
Collodium Schering-Kahlbaum "zur Herstellung von Membranen"	125	19	100	262
"Celloidin" Schering-Kahlbaum "for general use"	126	70	228	410
Collodium Schering-Kahlbaum DAB 6.	124	26	195	390

* This concentration was chosen because it gives maximum effects with membranes of moderate activity.[3]

different brands of collodion. Several other brands[7] of ether-alcohol soluble nitrocellulose not listed in the tables were also tested. Their behavior and their content of impurities are about the same as those found with the domestic brands of collodion obtainable from supply houses.

[7] For providing such samples of nitrocellulose we should like to express our thanks to the Hercules Powder Company and the American Cyanamid and Chemical Corporation.

Incidentally, it may be mentioned that Preuner and Roder,[8] investigating anomalous osmosis at about the same time as Loeb, used a Schering collodion, called "Kolloidin," for their experiments.

Though many casual remarks concerning our problems are contained in the membrane literature, only very few papers deal specifically with it. Most of the older work on collodion membranes assumes more or less tacitly that the electrical properties of such membranes are due to ion adsorption. This view, lately considered skeptically by many investigators, at least for strong electrolytes, could hardly furnish a basis for an explanation of our problem, namely, the differences between different brands of collodion.

Michaelis, in one of his latest papers on collodion membranes,[9] makes the following statement pertaining to our problem:

> "It must be left to a further study of the experts of collodion manufacture to investigate how this effect is influenced by the method of manufacturing. It certainly is not the degree of nitration. But after personal discussions with experts, I think it possible that the degree of degradation of the original cellulose molecule during the process of nitration is of importance. The specific effect seems to be greater the more intact the molecular size of the cellulose remains during nitration."

Wilbrandt, in considering this question, says "This difference is certainly not due to different sizes of pores, for the membranes with low potentials often had a high electric resistance and vice versa. Different degree of nitration does not seem, either, to be the cause." The NO_3-groups of the nitrocellulose—according to Wilbrandt—act as dipoles, with the negative charge directed towards the intermolecular spaces, *i.e.* towards the pores, thus causing in some way the charge of the membrane. "Now Mathieu," Wilbrandt continues, "has found that the rearrangement of the molecules in the films, especially in the highly nitrated ones, was very variable. Sometimes he obtained very sharp interferences, sometimes very indistinct patterns. It is highly probable that the variability of concentration potentials is due to this variability of the arrangement of the molecules."

Wilbrandt, in common with most earlier authors, bases his discussion of the collodion membrane and its behavior entirely on a consideration of the ideal nitrocellulose molecule. The ideal nitrocellulose molecule should be an inactive substance whatever its NO_3 content may be. However, it should be noted that collodion, *i.e.* ether-alcohol soluble nitrocellulose, is not cellulose hexanitrate but contains definitely less nitrogen. A detailed

[8] Preuner, G., and Roder, O., *Z. Elektrochem.*, 1922, **28**, 54.

[9] Michaelis, L., *Kolloid-Z.*, 1933, **62**, 2.

discussion of Wilbrandt's special views is beyond the scope of this paper. Suffice it to say that in our opinion it does not seem likely that dipoles should be able, in the manner indicated by Wilbrandt, to influence the charge of the membrane. The structure of an electrical double layer, particularly in the presence of considerable concentrations of electrolytes, can hardly be influenced to such an extent merely by oriented dipoles. The results of our investigations presented in this paper moreover make the hypothetical assumptions of Wilbrandt entirely unnecessary.

Meyer and Sievers,[10] emphasizing a point mentioned by several earlier investigators, believe that the electrical properties of collodion membranes are due to acid groups: "These acid groups could be carboxyl groups which are always found in cellulose, or possibly semi-esterified sulfate groups." At another place they mention pectic substances as possible carriers of acid groups.

That cellulose and cellulose derivatives generally have a certain degree of acidity seems to be universally acknowledged. Cellulose chemists usually discuss these properties in a casual way under the heading "oxycellulose."[11] A recent paper of Beutner, Caplan, and Loehr[12] deals specifically with the acidic properties of collodion. In a subsequent paper we hope to discuss this problem from a broader angle and a quantitative point of view. Whatever the outcome of these investigations will be, the general idea that impurities of an acidic nature cause the electrochemical activity of collodion lends itself to an experimental test. According to this general view, activity and degree of impurity should go parallel, the most active brands being the most impure ones.

It seems to be not without significance that the most inactive of the brands of collodion ("Parlodion" Mallinckrodt) tested by Wilbrandt and ourselves is also the most expensive one and that Kollodium Schering-Kahlbaum DAB 6 and "Celloidin for general use" are the least expensive grades among the Schering-Kahlbaum preparations.

A product like ether-alcohol soluble nitrocellulose (collodion) is undoubtedly variable in different respects as to degree of nitration, particle

[10] Meyer, K. H., and Sievers, J.-F., *Helv. Chim. Acta*, 1936, **19**, 649, 665.

[11] It is obviously outside the scope of this paper to discuss the very controversial matter of "oxycellulose." The interested reader is referred to the literature. Summaries may be found, *e.g.* in Schwalbe, C. G., Die Chemie der Cellulose, Berlin, Borntraeger, 1910/11 and 1918, particularly pp. 221 *ff.*, and Hess, K., Die Chemie der Zellulose und ihrer Begleiter, Leipzig, Akademische Verlagsgesellschaft, 1928, particularly pp. 455 *ff.*

[12] Beutner, R., Caplan, M., and Loehr, W. M., *J. Biol. Chem.*, 1933, **101**, 391.

size distribution and mean molecular weight, impurities, etc. These factors may all be interrelated to some extent.

Nevertheless, a better knowledge of the factors basically determining the electrochemical behavior of collodion should carry us a step further toward the solution of a problem which has puzzled many investigators in the past.

It may be worthwhile at this point to recall the conventional process of nitrocellulose manufacture, which in essence is as follows: Cellulose is swelled in NaOH, bleached if necessary with chlorine to obtain a colorless product; it is then washed, dried, and treated with a mixture of nitric and sulfuric acids. This product is freed from acid by washing, followed by prolonged boiling with a very dilute acid solution or with water. For products of a high degree of purity, this boiling process may be carried on for 100 hours or longer. From the rather indefinite statements in the literature one gets the idea that this very prolonged washing is necessary to hydrolyze certain sulfuric acid compounds, probably esters.

One, therefore, has to expect that collodion prepared from poor, unclean raw material would always yield an "active" collodion, since thorough bleaching would be necessary. We may safely assume that such bleaching would yield many oxidized groups, the end groups of the cellulose chain molecule and possibly CH_2OH-groups being acted upon. Sufficient oxidation would result in the presence of carboxyl groups on the nitrocellulose chain. These acid groups could be the factor determining the electrochemical properties of membranes prepared from such material.

Since oxidation is known to cause a splitting of cellulose chains, one would expect that such a product would have a lowered mean molecular weight. Consequently it should show low tensile strength in the dry state, as in the form of a film, and low viscosity when dissolved.

Unclean raw material may also contain appreciable quantities of pectic substances; after treatment with alkali, they would actually be hydrolyzed to pectic acid. Such substances would not be destroyed by nitration; indeed, nitropectin has been described as having properties generally similar to those of nitrocellulose.[13]

Finally, any material which is not sufficiently purified (whatever the purity of the raw cellulose used may be) could contain sulfuric acid in some combined form. If present as acid cellulose esters, it would make the collodion "active."

A possible experimental approach to our problem was to determine whether parallelism between "activity" and ash content is indicated in a

[13] Henglein, F. A., and Schneider, G., *Ber. chem. Ges.*, 1936, 69, 309.

comparison of different brands of collodion. This method involves the rather reasonable assumption that the non-volatile bases originating from the materials and water used are present somewhat proportional to the number of acid groups.

Dried samples of the various collodions were therefore carefully ashed. In order to avoid too rapid combustion, it was found practical to thoroughly wet the samples with a mixture of equal amounts of alcohol and water and to burn slowly in a covered platinum crucible, adding only small quantities of the nitrocellulose at a time.

TABLE III

Some Chemical and Physical Characteristics of Several Brands of Collodion

1	2	3	4	5	6
Brand of collodion	Mg. ash per gm. dry collodion	Optical properties of commercial solutions	Tensile strength of membranes	Viscosity (relative values, water = 3.7)	Mg. SO_4 per gm. dry collodion
"Parlodion" Mallinckrodt	0.16	Clear*	Very high	106	0.04
Collodion Merck U.S.P.	0.23	Clear	Very high	93	0.2
Collodion Baker U.S.P.	0.45	Clear	Very high	82	0.2
Collodium Schering-Kahlbaum "pro analysi"	0.4	Very slightly turbid	High	88	0.3
Collodium Schering-Kahlbaum "zur Herstellung von Membranen"	1.3	Turbid, small sediment	Poor	40	0.9
"Celloidin" Schering-Kahlbaum "for general use"	3.6	Very turbid, sediment†	Poor	46	2.0
Collodium Schering-Kahlbaum DAB 6	3.5	Very turbid, heavy sediment	Very poor	28	3.1

* The solid commercial product yields a clear solution in ether-alcohol.

† The commercial product yields a very turbid, strongly yellowish solution in ether-alcohol; some sediment appears on standing.

The results of these analyses are summarized in column 2 of Table III. A comparison with Table I and Table II reveals an obviously close parallelism between activity and ash content.

Columns 3, 4, and 5 of Table III give a comparison of other properties, namely, optical properties of the commercial solutions, tensile strength, and viscosity.

The domestic brands of collodion solutions are perfectly clear and

usually show no appreciable scattering of light. With the four grades of Schering-Kahlbaum collodion used there is a close parallelism between increasing turbidity and tendency toward sediment formation on the one hand and ash content on the other. Only Collodium Schering-Kahlbaum "pro analysi" was somewhat similar to the purer brands of collodion.

The tensile strength of membranes (of roughly the same thickness) was high for the three brands named first in Table III. It was somewhat less for Collodium Schering-Kahlbaum "pro analysi" and increasingly less for the next two preparations. Collodium Schering-Kahlbaum DAB 6 showed very poor strength.

The viscosity determinations were made with 5 per cent solutions of (previously dried) *collodion in a mixture of equal parts of absolute ether and absolute alcohol. The time required for the meniscus to pass two marks on a 5 ml. pipette used as viscosimeter was* measured. The experimental conditions were such as not to cause any complications due to evaporation. The water value of our pipette viscosimeter was 3.7 seconds. The time values found with the different collodions are given in column 5 of Table III.

Low tensile strength and low viscosity in solution are both strong indications of a lower molecular weight. The remarkably close parallelism between the several properties of these collodions cannot reasonably be considered to be fortuitous. However, it may be added that an exact quantitative correlation is impossible because we are dealing with too many variables.

The experimental results reported so far are in good agreement with the views outlined above. The more active grades of collodion are the poorer ones technically speaking, containing many impurities of an acid character; their mean molecular weight, moreover, is obviously considerably less, as can be readily concluded from the tensile strength and viscosity data.

Partial oxidation could readily account for the presence of acid groups and likewise for a lower molecular weight, as such an oxidative breakdown of cellulose is a well established fact.

This otherwise satisfactory explanation has not heretofore taken into account the possibility of the presence of acid sulfuric acid compounds in the collodion. Such compounds could cause all or part of the observed electrochemical activity, though their presence could not account for the lower molecular weight. The parallelism between activity and degradation of the nitrocellulose molecules would then be entirely accidental.

Our next step, therefore, was to determine whether or not the different brands of collodion contain sulfate, and if so, how much.

Collodion samples were ashed carefully as outlined above. The residues were analyzed and found to be substantially a mixture of aluminum, iron, and calcium sulfate, containing very little free base and no detectable quantities of silicic acid. The analytical results, however, indicated that a loss of sulfate may occur because of a lack of fixed alkali. To obtain correct sulfate values, therefore, the experiments were repeated with a changed technique. The collodion samples were thoroughly wetted with an alcoholic solution of sodium hydroxide and ashed after this treatment. The sulfate values found were accordingly higher than those obtained with the former technique. The results are listed in column 6 of Table III. These latter values represent in our opinion the true sulfate contents of the different collodions.[14]

These data show that the sulfate content also runs strictly parallel to the electrochemical activity of the different preparations. In order to cause activity the sulfate present must necessarily be in the form of an acid compound. Sulfuric acid esters of cellulose have been described repeatedly and their appearance in improperly purified nitrocellulose seems to be assumed quite generally.

However, it may be possible that the sulfate, as found by analysis, is present in a combined though inactive form, or merely as an admixture originating from the process of manufacture. How difficult it is to remove sulfuric acid from nitrocellulose can be gathered from the following experiment.

A sample of collodion (Merck U.S.P.) with a sulfate content of 0.2 mg. per gm. of dry material was dissolved in acetone. A solution of sulfuric acid in acetone was added until the mixture was about 0.1 molar with respect to sulfuric acid. After 2 hours the solution was allowed to drip slowly under stirring into a great excess of water. The fibrous product obtained was washed thoroughly and boiled for 35 hours with water. After washing and drying, an ether-alcohol solution was prepared and centrifuged to remove all insoluble particles and the sulfate content of this purified material was determined as before. Its sulfate content of 0.5 mg. per gm. dry collodion is more than twice that determined in the original material.

Membranes cast in the usual manner from ether-alcoholic solutions of this treated collodion showed no significant change in activity as compared with the original material. The possibility, therefore, is not entirely excluded that at least some of the sulfate content of collodion is present as inactive admixture, particularly in the more active, poorly purified products.

We are still confronted with the apparent correlation between sulfate

[14] The conventional technical method of sulfate determination after Berl (*cf.* Berl-Lunge, Chemisch -technische Untersuchungsmethoden, Berlin, Julius Springer, 1934, 5, 735) does not seem readily applicable to the very small amounts of sulfate with which we had to deal.

content and activity in the commercial preparations; sulfate groups may be responsible for at least some of the activity of collodion, though the relative importance of this factor is still an open question.

There are two apparent ways to estimate the relative importance of sulfuric acid compounds for the activity of collodion. The most straightforward and theoretically simplest approach would be to compare the sulfate content with the total number of acid groups present in the collodion. Seemingly simple, it is beset with experimental difficulties. We, therefore, must postpone its discussion to a later paper, in the frame of which it will find a more proper place.

The other method would be to investigate the possibility of freeing active collodion of its sulfate content without total loss of activity.

Our plan, therefore, was to reduce in some way the sulfate content of active collodions down to the level of the more inactive brands and to see whether or not their activity is lost with the sulfate removal.

According to the literature, the desired purification could be tried in several ways.[15] One could boil a sample for a long time in very dilute acid and water, as is done in the conventional commercial process. This procedure is supposed to hydrolyze sulfuric acid compounds. Furthermore, one could accelerate the hydrolysis of the assumed sulfuric acid esters by boiling with half concentrated acetic acid.

In judging the experiments described below, one has to consider that on boiling, not only sulfuric acid compounds may be hydrolyzed and thus rendered inactive, but also that any ionizable compound may be slowly dissolved. Once dissolved, such compounds would be largely lost in the boiling liquid. This is likewise true for oxidation products and acid esters which probably are not too different in their general properties.

We were able to obtain an adequate supply of only two of the more active brands of collodion, namely, "Celloidin, Schering-Kahlbaum, for general use," a very active preparation, and "Collodium, Schering-Kahlbaum, zur Herstellung von Membranen," a moderately active preparation.

To make them suitable for the intended purification, their rather dilute ether-alcoholic solutions were poured slowly into a great excess of water under continuous, vigorous stirring. The products so obtained are composed of fine fibres.

Boiling in water turned out to be a somewhat less suitable method for our purpose. Therefore, since we had to economize on our material, this method was applied only to one brand of collodion, "Celloidin, Schering-Kahlbaum, for general use." 30 gm. of Celloidin were treated in about 1.5 l. of liquid. The sample was heated every morning

[15] See *e.g.*, Hess, K., Die Chemie der Zellulose und ihrer Begleiter, Leipzig, Akademische Verlagsgesellschaft, 1928, 367, 381, etc.

and allowed to boil for about 6–7 hours; on the next morning the liquid was renewed. The boiling liquid used for several days was very dilute hydrochloric acid, later on, distilled water. The water was replaced less frequently. After a boiling time of about 70 hours, the sulfate content was reduced to the desired level. The material was then thoroughly dried. The sulfate determination was carried out as described previously. An ash determination was also made.

The second method of purification was applied to both of our available active preparations. 12 gm. samples were boiled in about 700 ml. of 60 per cent acetic acid. After an appropriate time, the boiling was stopped and the material thoroughly freed of acetic acid by repeated boiling and thorough washing with water. It was finally dried. The boiling time necessary to reduce the sulfate content to the desired level was 10 hours for Collodion Schering-Kahlbaum "zur Herstellung von Membranen" and 16 hours for "Celloidin Schering-Kahlbaum for general use." The dry materials were analyzed for ash and sulfate content as in the preceding case.

In Table IV*c* the analytical data pertaining to our purified collodions are given. They are preceded by the corresponding data of active commercial collodions—Table IV*a*—and pure, inactive commercial collodions—Table IV*b*.

To compare the "activity" of the different preparations we used data concerning anomalous osmosis and concentration potentials obtained with the same membranes. Anomalous osmosis, as said above, is a very sensitive indicator as to the activity of such membranes.

Membranes of the original and purified collodions (dissolved in 75 per cent ether-25 per cent alcohol) were cast; the first three of each brand giving proper rises with sugar solution after 20 minutes (column 4 of Table IV) were used. They were all of about the same porosity. The anomalous osmosis experiments were carried out as indicated before. The corresponding manometric rises are listed in column 5 of Table IV. Column 6 gives the concentration potentials between 0.01 N and 0.02 N KCl solutions for the same membranes.

On examination of the facts summarized in Table IV, one sees that there is no necessary correlation between sulfate content and activity. Though the originally active brands of collodion lose some of their activity in the process of purification, they still are much more active than the better commercial preparations of the same sulfate content. We would prefer to attribute the decrease in activity on purification largely to a loss of active, but sulfate-free, substance. As said above, such compounds are, in our opinion, partially oxidized nitrocellulose molecules carrying carboxyl groups and having probably on the average a lower molecular weight and a much greater solubility than the more perfect, less oxidized nitrocellulose molecules. This view is substantiated by the fact that the filtered boiling liquids leave on evaporation a considerable residue of organic substance.

TABLE IV

Sulfate Content and Activity of Some Commercial and Purified Collodion Preparations

1	2	3	4	5	6
Brand of collodion	Mg. ash per gm. dry collodion	Mg. SO_4 per gm. dry collodion	Osmotic rise with sugar $\frac{M}{4}$	Anomalous osmotic rise with K_2SO_4 $\frac{M}{512}$	Concentration potential KCl 0.01 M/KCl 0.02 M
Table IV a					
			mm.	*mm.*	*mv.*
Collodion Schering-Kahlbaum "zur Herstellung von Membranen" (commercial preparation)	1.3	0.9	106 118 130	85 98 104	1.4 1.4 1.2
"Celloidin" Schering-Kahlbaum "for general use" (commercial preparation)	3.6	2.0	110 126 130	190 228 244	2.2 3.6 2.0
Table IV b					
			mm.	*mm.*	*mv.*
Collodion Merck U.S.P. (commercial preparation)	0.23	0.2	116 125 138	48 46 57	0.5 0.8 0.9
Collodion Baker U.S.P. (commercial preparation)	0.45	0.2	128 130 136	32 47 50	1.0 1.1 0.5
Table IV c					
			mm.	*mm.*	*mv.*
Collodion Schering-Kahlbaum "zur Herstellung von Membranen" (purified with acetic acid)	0.2	0.2	112 126 130	84 98 96	2.0 1.1 1.4
"Celloidin" Schering-Kahlbaum "for general use" (purified by boiling in water)	0.2	0.2	118 136 150	72 93 108	1.3 2.1 1.7
"Celloidin" Schering-Kahlbaum "for general use" (purified with acetic acid)	0.2	0.2	118 130 138	102 126 120	2.6 2.3 2.3

We are, therefore, very much inclined to think that the electrochemical activity of the more active collodion preparations is mainly due to sulfate-

free acidic compounds, sulfate-containing substances playing only a minor rôle.

GENERAL DISCUSSION

The evidence presented in the preceding pages integrates itself into a relatively simple picture.

The electrochemical "activity" of collodion is due to impurities of an acidic nature originating from the raw material and the manufacturing process. The purest brands of commercial collodion show very low activity. Therefore, the assumption that nitrocellulose as such is electrochemically very inactive[16] seems justified.

The electrochemically active impurities are substantially not sulfuric acid compounds, as borne out by the fact that active commercial collodions can be largely freed from their sulfate content without parallel loss of activity.

This leads us to the conclusion that carboxyl groups must be responsible for the observed activity. Carboxyl groups are undoubtedly contained in all cellulose raw materials.[17] Their number must be greatly increased by bleaching, particularly the excessive bleaching necessary with inferior raw material. Excessive bleaching yields products of lowered molecular weight; *i.e.*, low viscosity in solution and low tensile strength of films.

To obtain a true picture it is impossible to think of nitrocellulose in terms of ideal uniform molecules. Nitrocellulose is in reality composed of mole-

[16] This undoubtedly applies to other cellulose derivatives, esters as well as ethers and also to straight cellulose and hydrocellulose.

The use of such membranes seems to offer some hope for successfully attacking the old experimental problem of measuring directly the osmotic pressure of electrolyte solutions. Investigators in this field have employed membranes which, it would seem, are particularly apt to give anomalous osmosis. We know; *e.g.*, that Cu-ferrocyanide is a very active membrane (Bernstein, Elektrophysiologie, Braunschweig, Friedrich Vieweg and Sohn, 1912, 164). The same is true for silicate membranes and probably many other membranes of salt character (Grollman, A., and Frazer, J. C. W., *J. Am. Chem. Soc.*, 1923, **43**, 1710; Grollman, A., Dissertation, Johns Hopkins University, 1923; Sollner, K., and Grollman, A., *Z. Elektrochem.*, 1932, **38**, 274; *Tr. Electrochem. Soc.*, 1932, **61**, 477, 487). The problem seems to be to find a membrane with so low a charge density that the electrical forces become negligible. It may be recalled here that Loeb was able to reduce practically to zero the activity of his proteinized collodion membranes simply by working at the isoelectric point of the particular protein used (Loeb, J., *J. Gen. Physiol.*, 1920, **4**, 463).

[17] See *e.g.* Hess, K., Die Chemie der Zellulose und ihrer Begleiter, Leipzig, Akademische Verlagsgesellschaft, 1928, particularly pp. 54 *ff*.

cules of very different length and these molecules are by no means all perfect. Many of them carry—and this is the point important for us—carboxyl groups, some sulfate groups, and possibly some others. Thorough purification gradually removes these ionizable impurities.

The best grades of collodion, therefore, are composed of long fairly ideal nitrocellulose molecules, probably carrying only here and there a group (*e.g.*, carboxyl) not compatible with the ideal structure. Correspondingly, they are fairly inert.

Collodion purified to a lesser extent in any case contains many impurities, largely of lower molecular weight.

Collodion prepared from inferior raw material is composed of molecules of lower mean molecular weight.[18] If not extremely well purified, such a material would be expected to contain many molecules carrying oxidized groups which originate from excessive bleaching.

This picture fits the three inferior grades of Schering-Kahlbaum[19] collodion perfectly. The high sulfate content is obviously the result of a more superficial purification.

The correctness of our explanation of the electrochemical activity of collodion is further substantiated by the fact that collodion, as noted in a preceding paper,[2] can be activated by oxidation.

The results here obtained are somewhat unexpected and indeed ironical. The brands of collodion preferred by nearly all workers in the field of electrochemical membrane investigations are, technically speaking, the poorest ones.

If one tries to visualize the molecular mechanism of electrochemical membrane activity, one likewise arrives at a relatively clear picture. Without entering at this point into any discussion on the relative merits of different permeability theories (*i.e.*, pore *versus* homogeneous phase hypothesis) as applied to the collodion membrane, we shall employ the pore conception for the following discussion. It allows us to discuss without much distinction the highly dried (controversial) and the incompletely dried, undoubtedly porous collodion membranes.

As pointed out by several investigators, recently in a more quantitative form,[10, 20] the electrochemical behavior of such a membrane depends upon the relative number and mobilities of all the ions present in the pores.

[18] We are quite aware of the fact that low molecular weight does not necessarily have its origin in either poor raw material or oxidation. Such material is actually manufactured widely for special technical purposes from high grade raw material, special processes being used to reduce the molecular weight.

[19] Unfortunately, several letters sent to the Schering-Kahlbaum Company requesting information on this problem remained unanswered.

[20] Teorell, T., *Proc. Soc. Exp. Biol. and Med.*, 1935, **33**, 282.

Some of the ions of one sign are fixed to the wall, their "gegen-ions" being freely movable in the aqueous phase. In very wide pores, or in pores of any size, if only a negligible number of ions is fixed to the pore walls, the specific membrane influence is negligible; high electrolyte concentrations in the aqueous phase likewise reduce the relative importance of the wall influence. In narrower pores and in more dilute electrolyte solutions, if by some means the relative number of ions fixed immovably to the pore walls is increased, the specific membrane influence increases. For our specific problem we are concerned only with the latter factor and the question is: what determines the number of ionizable points fixed immovably at a collodion/aqueous solution interface?

This situation with respect to the membrane (ϵ) potential applies *mutatis mutandis* quite as well to the electrokinetic (ζ) potential.

The older assumption of preferential ion adsorption has recently been questioned because of the extremely low adsorbability of strong inorganic electrolytes.

Our results also indicate that ion adsorption has only a very secondary, if any, influence on the electrochemical activity of collodion membranes in such solutions.

Lately, several investigators have assumed that the ionizable groups on collodion in strong electrolyte solution belong to the collodion itself. This view seems to be proven by our experiments. It seems much more familiar when one recalls the structure and behavior of proteinized membranes. In this case, everybody agrees that all the electrochemical properties of the membrane are due to the ionizable groups of the protein.

The great differences between different collodion preparations are now easily understood. The purest ones carry only a small number of dissociable groups and are, therefore, inactive. The less carefully prepared material contains many acidic groups and is electrochemically active. The number of ionizable groups per unit of area is an inherent property of the membrane material used. In any given solution, their dissociation, *i.e.* their actual effectiveness, depends on the nature and concentration of all the ions present, particularly the possible gegen-ions.

In our opinion the behavior of weakly adsorbable polyvalent ions, *e.g.* sulfate, has to be explained on the basis of a combination of mechanical and electrical sieving effects.

This conception, of course, does not apply without restriction to all situations. For example, if any of the ions present in solution is very strongly adsorbable, it must strongly influence the ionic build-up of the interfacial layer. In this case we can undoubtedly approach the situation formerly assumed also for strong electrolytes.

It is conceivable that with a membrane material which is completely or nearly completely void of any dissociable groups, even in solutions of strong electrolytes, preferential ion adsorption comes into play to a decisive extent. However, no case of this nature has so far been described for collodion membranes.

Previous mention was made of some of the factors which may possibly have a great influence on membranes cast from different collodions. They are degree of nitration, impurities, mean molecular weight, and particle size distribution. To this we may add the solvent used. The degree of nitration obviously (as pointed out by earlier investigators) is not a decisive factor, as attested by the fact that nitrocellulose of widely varying nitrogen content yields membranes having quite similar electrochemical properties. The paramount importance of impurities for the electrochemical behavior of collodion membranes is discussed in the preceding pages. Concerning the mean molecular weight and particle size distribution and the influence of the solvent, we are inclined to believe that these factors are quite intimately connected in a consideration of the spatial structure of membranes. Together, they probably determine the geometrical arrangement of the molecules in the films. We do not intend to discuss at this time this extremely complex problem since our present results have very little bearing upon it.

In subsequent papers we propose to discuss the preparation of artificially activated membranes and to investigate quantitatively the acidic properties of collodion, hoping that it may be possible to correlate such data with some of the newer theoretical considerations of electrochemical membrane behavior.

SUMMARY

1. The electrochemical behavior of membranes prepared from commercial collodion preparations varies widely, some preparations showing very high, other ones very low electrochemical efficiency ("activity").

2. The electrochemical activity of a collodion membrane depends entirely upon impurities of an acidic nature contained in the collodion used for casting the membrane.

3. The active acidic impurities are substantially due to partial oxidation which occurs in the manufacturing process. Sulfuric acid compounds; *e.g.*, acid sulfuric acid esters play only a minor rôle, if any.

4. The electrochemical behavior of collodion membranes in solutions of strong electrolytes is decisively dependent upon the acidic groups built permanently into the collodion surfaces. Preferential ion adsorption plays only a minor, if any, rôle.

MELANOPHORE BANDS AND AREAS DUE TO NERVE CUTTING, IN RELATION TO THE PROTRACTED ACTIVITY OF NERVES

By G. H. PARKER

(*From the Biological Laboratories, Harvard University, Cambridge*)

PLATES 1 AND 2

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1. INTRODUCTION

Brücke (1852), in his study of the color changes of the African chameleon, was the first to record the fact that the cutting of chromatic nerve fibers was followed by a darkening of the area of skin thus denervated. He ascribed this condition to paralysis. Pouchet (1876) also recorded such a darkening in fishes, particularly in turbots, and accepted Brücke's interpretation of it. Von Frisch (1911) added to the list of fishes that showed these peculiarities and noted incidentally that 8 days after the initiating operation the dark area thus formed on the fish began to show signs of blanching and that in 13 days it was strikingly pale. The blanching of such dark areas was confirmed on the minnow *Phoxinus* by Smith (1931) who pointed out that the ultimate loss of color in these areas was possible only when the fish was maintained in a relatively pale state. Meanwhile Wyman (1924) had used denervated bands on tails, now generally known as caudal bands, for an extended study of chromatic physiology in fishes, and the technique of the caudal band came into common use among numerous workers (Abolin, 1925; Smith, 1928; Fries, 1931; Mills, 1932; Matthews, 1933; Parker and Porter, 1933; Abramowitz, 1935; Kleinholz, 1935; Foster, 1937; Dalton and Goodrich, 1937; Kamada, 1937; Odiorne, 1937; Wykes, 1938; Osborn, 1938; Matsushita, 1938; Tomita, 1938; Vilter, 1938; and others).

A little over half a decade ago it was pointed out that when in a fully blanched caudal band on such a fish as *Fundulus* a new cut was made strictly within the limits of the original band and slightly distal to the first cut, the dark tint of the original band would reappear though not so fully as at first (Parker, 1934 *a*). This condition was of prime importance in two respects. It showed, first, that the chromatic nervous mechanism of the band was not paralyzed, as had been assumed since the days of Brücke, but that it was merely quiescent and could easily be reactivated. And it showed further that the darkening of the band was not the result of checking

certain assumed central influences whereby the melanophores had been held contracted (Zoond and Eyre, 1934; Zoond and Bokenham, 1935; Sand, 1935; Wykes, 1938), but that this darkening resulted from an unusual stimulation of the dispersing nerve fibers at the site of the wound. This new interpretation has been called the superactivity hypothesis as contrasted with the older one which was designated as the paralysis hypothesis (Parker, 1936). Since a caudal band in *Fundulus* may remain visible for hours or even a day or more after its first formation it was suggested that the dispersing nerve fibers in such a band are active over a correspondingly long period, a view that has met with very little approval from other workers. The possibility of long continued activity in such nerve fibers will be considered in the following pages and this question will be discussed not only from the standpoint of caudal bands, but from that of the larger areas of skin darkened by denervation.

The technique employed in these studies calls for no special description. Where operations of considerable extent were performed on the fishes the creatures were anesthetized by cold in water and cracked ice (Parker, 1939), a method which proved to be in every way satisfactory.

2. *Revival of Bands and Other Areas*

Faded caudal bands in *Fundulus* are readily revived by recutting. In Fig. 1 is shown the tail of a moderately pale *Fundulus* on which 3 days previously a caudal band had been formed by cutting a ray near the root of the tail (lower band in the figure). After this band had fully blanched it was recut distal to the position of the initial cut with the result that the band was revived though with less intensity of shade than that of the original band. At the same time that the second cut was made a ray slightly above the middle of the tail was also cut and a strong caudal band was thus produced as shown in Fig. 1. This new band agreed in intensity with that of the first band when it was originally cut and could be used as a check on this band. The condition of the melanophores as seen in the two bands and the normal tail in Fig. 1 are shown in Figs. 2, 3, and 4. In the normal tail (Fig. 2) the melanophore pigment is nearly though not completely concentrated, a condition characteristic of a fairly pale fish. In the newly formed band (Fig. 3) the melanophore pigment is almost fully dispersed and in the recut band (Fig. 4) this dispersion is pronounced though not so extensive as in the newly formed band. Thus the gross appearance of the bands in the tail as well as the condition of the pigment in the three states of the melanophores agree in showing that a caudal band in *Fundulus* may be revived by recutting though the band thus reactivated is never so intense in its revived state as in its initial one.

The revival of a band in *Fundulus* is not only thus possible but this process may be repeated in this fish at least twice. One example will suffice. In a pale *Fundulus* in which a caudal band had blanched in about 11 hours the band was revived by recutting and was again blanched in about a day after which on a renewed cutting a third darkening took place. This third response was by no means so pronounced as the second, but it was visible beyond a doubt. A fourth attempt failed to elicit an unquestionable redarkening. This failure was probably due to the beginning of degeneration in the chromatic nerve fibers which makes itself manifest usually in about 5 days after the initial cut (Parker and Porter, 1933). A simple reactivation of bands such as occurs in the melanophore system of *Fundulus* has also been observed in the erythrophore system of the dorsal fins in the squirrel-fish *Holocentrus* (Parker, 1937).

The revival of blanched caudal bands in the catfish *Ameiurus* as described by me some years ago (Parker, 1934 *b*) has been questioned by Wykes (1938) and by Osborn (1938). Osborn in particular stated that although he recut faded bands in this fish repeatedly he never was able to produce a second darkening. I reinvestigated this matter (Parker, 1940) and found that faded bands in both normal and hypophysectomized catfishes could be readily revived by recutting provided the fishes tested were kept in water at ordinary summer temperatures, about 20°C. I attributed Osborn's failure in this respect to the low temperature at which he had worked, 12°C. In a recent retesting of catfishes in water at this degree of cold I too was unable to obtain reactivation of bands. This is in line with Wykes' statement (1938) that at the winter temperature of 6°C. the color activities of *Ameiurus* are in almost full abeyance.[1] The revival of blanched caudal bands, though not without exception, has been reported for the Japanese catfish *Parasilurus* by Matsushita (1938). Catfishes are less satisfactory for testing color revival than *Fundulus* probably because of the slowness with which their bands blanch. This step in *Ameiurus* often takes some days and before the bands are pale enough to be recut their nerves have probably begun to degenerate and are thereby rendered incapable of response. This I suspect may be the reason Vilter (1938, 1939 *a*, 1939 *b*) was unable to reactivate the bands in the dorsal fins of *Gobius*, though here too temperature may play a part. Unfortunately this worker makes no statements as to the details of his procedure. Certainly the overlapping

[1] The effect of differences in temperature on the chromatophore changes in *Macropodus* has recently been recorded by Dalton and Goodrich (1937) who observed that the blanching of dark caudal bands in this fish required more time (10–18 hours) at 20°C. than at 29°C. (5–8 hours).

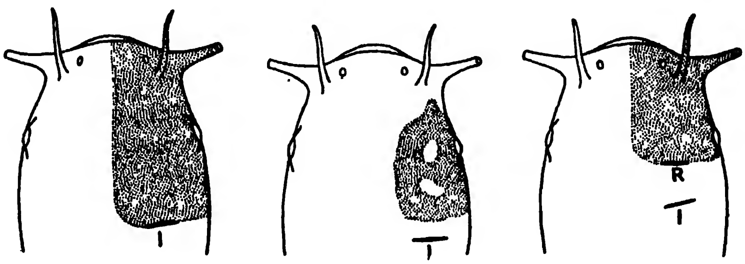
of the areas of distribution of the chromatic nerve bundles which he has observed in the dorsal fins of *Gobius* can offer no explanation for the revival of caudal bands in *Ameiurus*, for no such overlapping occurs in the tail of this fish (Parker, 1934 *b*). This in fact is one of the advantages of *Ameiurus* for this type of work.

Although caudal bands and other like areas appear to be open to reactivation wherever they are properly tested, it is quite unknown, so far as I am aware, what occurs in the larger areas of skin darkened by denervation. Such areas as these, however, often covering a considerable part of a fish, were what called the attention of the earlier investigators to this subject. By cutting an appropriate bundle of nerves much of the head of a *Phoxinus* can be darkened (Smith, 1931), or a quadrant of the body of a *Fundulus* (Parker, 1936), or even the posterior half of a *Macropodus* (Kamada, 1937). Will these large areas blanch and are they open to the kind of revival that has been shown to occur in caudal bands?

For convenience in this kind of experimentation the head regions of such fishes as *Fundulus* and *Ameiurus* are very satisfactory and the cranial nerve best adapted for cutting in this part of the fish is the ophthalmic. When in a pale *Fundulus* the orbit is opened dorsally and the ophthalmic nerve which lies on the roof of this cavity is severed, the corresponding half of the head from the snout to the eye and from the lateral wall to the mid-dorsal line darkens within about a minute. This darkening increases for half an hour after which it gradually blanches until in a day or so it has largely disappeared. It vanishes not by fading throughout its whole expanse, but by shrinking on its edges till the last part that is visible is a small dark patch dorsal to the eye. When this experiment is carried out on fully dark killifishes, no change in the tint of their heads is to be noticed, for the heads of such fishes like the rest of their bodies are from the beginning very dark. If in a pale *Fundulus* the ophthalmic nerves of both sides of the head are cut simultaneously, the head as a whole darkens after which it slowly and completely blanches. These observations on *Fundulus* substantiate fully those of Smith (1931) on *Phoxinus*.

Similar tests on *Ameiurus* can be carried out with greater convenience because of the large size of its head. The responses of *Ameiurus*, however, are relatively slow. If the ophthalmic nerve in a pale *Ameiurus* is cut by penetrating at an appropriate position the dorsal bony wall of the orbit with a narrow knife-blade, the half of the head concerned will begin to darken in about 10 minutes and will be fully dark in from 4 to 5 hours (Text-fig. 1). After this the dark area will commence to lose its deep tint and in 30 to 50 hours will have become about as pale as the rest of the fish. The blanching of this large cephalic area in *Ameiurus* shows details which

are not visible in *Fundulus*. The large cephalic area in *Ameiurus* shrinks toward the eye as it does in *Fundulus*, but with greater irregularity of outline. Before, however, the area in *Ameiurus* has diminished greatly one or two pale spots appear within its contour (Text-fig. 2). These eventually coalesce and sooner or later unite with the shrinking irregular outline and thus contribute to the gradual disintegration of the area as a whole. In this respect the disappearance of the area in *Ameiurus* is unlike that in *Fundulus* and very unlike the disappearance of caudal bands in either fish where the operation is strictly a shrinkage from the periphery.



1 2 3

TEXT-FIGS. 1, 2, and 3. Diagrams of the dorsal aspect of the head of a pale catfish, *Ameiurus nebulosus*, showing darkened areas resulting from the cutting of the ophthalmic nerve.

TEXT-FIG. 1. A catfish head showing the extent of the darkened area (stippled) that resulted from the severance of the ophthalmic nerve through the initiating aperture I.

TEXT-FIG. 2. The same catfish head as shown in Text-fig. 1 a day or two after the initiating cut had been made and showing the shrinkage of the original dark area by lateral invasion and by internal disintegration.

TEXT-FIG. 3. A catfish head like that shown in Text-fig. 1 whose dark area having been completely blanched was partly revived by a second and more distal cutting (R) of the ophthalmic nerve.

In *Ameiurus* as in *Fundulus* the simultaneous cutting of both ophthalmic nerves is followed by a darkening of the whole dorsal aspect of the head. Such very large areas in *Ameiurus* disappear also by irregular internal disintegration as has been described for the darkened half-cephalic area in this fish. From these observations on the killifish and the catfish I conclude that large areas darkened by nerve cutting in pale fishes can blanch as fully as caudal bands can.

Are these large areas open to revival of coloration as are caudal bands? To test this catfishes about 15 cm. long were much more favorable than killifishes which at a maximum were about half that length. By a transverse, vertical cut in the head of a pale catfish close to the region of the internal ear and on the exterior of the cranium, it was possible to sever the strand of autonomic nerve fibers supplied from the posterior part of the

system to the anterior cranial nerves. As a result of such a cut the head soon darkened by melanophore expansion and then in a few days blanched. If, when this stage had been reached, a second cut was made anterior to the first one and so that the ophthalmic nerve in the posterior part of the orbit was severed, the chromatic fibers for the top of the head could thus be cut for a second time. As a result of this the half of the head in front of the new cut began to darken in about 30 minutes after which the darkening continued for an hour or more till this somewhat smaller area was again decidedly dark (Text-fig. 3). This test was carried out on five catfishes all of which gave essentially the same result. In some the blanching of the first area came more quickly than in others and in some the second darkening was more pronounced than in others, but in all the initial dark area blanched in course of time and a final second darkening was always induced. I therefore conclude that large areas darkened by the severance of chromatic nerves such as the cephalic areas of the catfish not only blanch but may be revived by the recutting of their nerves as is true of caudal bands.

Other parts of catfishes on which somewhat similar tests can be made are the pelvic fins. The advantage of these fins for such work was first pointed out by Wykes (1938). These fins, which are situated on the ventral aspect of the fish immediately anterior to the cloaca, consist of a very transparent membrane supported by some eight fin rays (Fig. 5). They are provided with a sparse, rather uniformly scattered supply of well defined melanophores (Fig. 6) and are convenient for study in that the fin of one side of the fish may serve as a convenient control for that on the other side. When a ray in the pelvic fin of a pale fish is cut, a dark band forms (Fig. 7) as in the case of the tail. The pigment in the melanophores of such a band is fully dispersed (Fig. 9) as contrasted with that of the color cells in the rest of the fin (Fig. 8).

Each pelvic fin is innervated by some six spinal nerves which can be easily traced from near the vertebral column over the inner face of the body wall and into the fin. By a single longitudinal cut through the body wall to the body cavity slightly dorsal to the root of the fin all these nerves can be severed. Such a cut, about a centimeter long, can be easily closed by a few stitches. Fishes thus operated upon live well, remain active, and have been kept in aquaria for 10 days or more.

When one pelvic fin of a catfish is thus denervated, it soon darkens and remains dark for some days. This circumstance led Wykes to remark that it is difficult to ascribe this persistent melanophore expansion to an injury discharge in the severed fibers since it lasts unchanged for so long a time. She was therefore led to assume another explanation, namely, that the cut

acted not by excitation but by the elimination of some central activity such as inhibition. A simple experiment would have shown the error of this view.

In my tests on *Ameiurus* a denervated pelvic fin was found to begin darkening in from 5 to 10 minutes. In 2 hours or so it was fully dark and in 3 to 4 days it was blanched almost to the same degree of paleness as that of the fin of the opposite side. It was now comparatively easy to cut in the blanched denervated fin a single ray with its nerve and to ascertain thereby whether reactivation was possible. In all five fishes thus tested dark bands developed about the newly severed rays in strong contrast with the paleness of the fin as a whole (Fig. 10). The pigment in the melanophores of these bands was obviously though not extremely dispersed (Fig. 12) as compared with that in the melanophores of the other parts of the fin (Fig. 11).

Such darkened bands, which were observed in fishes kept at about 20°C. could have depended in no sense upon the elimination of central influences, for, if there were such, they had already been excluded by the first cut. The darkening as induced by the second cut must have originated in the cut itself. In this respect the pelvic fins of *Ameiurus* respond to chromatic tests in the same way as the caudal fins of this fish do. Thus all the evidence from nerve cutting including that from the pelvic fins, from the cephalic areas, and from the caudal bands, leads to one conclusion, namely, that when nerves with dispersing chromatic fibers in them are cut these fibers are not at once paralyzed but are especially activated whereby the melanophores associated with them are induced to disperse their pigment and thus to darken the denervated area.

3. *Is the Activity of Cut Dispersing Nerve Fibers Protracted?*

It was my original opinion (Parker, 1934 *a*) based upon a study of the dispersing chromatic nerves in the tail of *Fundulus* that after severance, these nerves were excited from the region of the cut distally for the approximate period during which the dark caudal band was clearly visible. This interval varied from a dozen or more hours to several days. In the Japanese catfish, as pointed out by Matsushita (1938), a caudal band may be present for more than 2 weeks, a very long time over which to assume the continuous activity of a nerve. Matsushita was therefore disposed to regard my suggested explanation as premature, and it is true that since this question was first discussed a number of new and significant observations have been made on the mechanism of color changes in fishes.

It is now generally admitted that at least three elements are involved in the chromatic responses of catfishes. These are the pituitary gland, and

two sets of autonomic nerve fibers one dispersing and the other concentrating. These three are very probably not the only elements concerned, but they are unquestionably the chief ones. From the pituitary gland is derived the blood-borne, dispersing neurohumor intermedin, from the concentrating nerves adrenalin, and from the dispersing nerves acetylcholine (Chin, 1939; Chang, Hsieh, and Lu, 1939; Parker, 1940). Osborn (1938) has shown that intermedin can be identified in the blood of a catfish in physiologically significant amounts some 70 hours after the loss of the pituitary gland. So far as color changes are concerned this substance disappears from the blood of hypophysectomized catfishes within 5 days after the operation. These observations show that intermedin is a reasonably stable and persistent agent. Adrenalin is known to remain active in the blood of the catfish for some hours after injection. On the other hand acetylcholine unprotected by such substances as eserine is destroyed almost at once in the circulation of this fish. Both acetylcholine and adrenalin, however, if carried in olive oil, may be introduced subcutaneously into catfishes in the form of coarse emulsions and under such circumstances these agents will remain effective as color activators for several days. In oil acetylcholine and adrenalin are evidently protected from destruction, and their action is thus prolonged (Parker, 1940). Under natural conditions they probably enjoy a similar protection and extension of activity by residence in the lipoid materials of the skin. Is it possible that the experimental extension of the color phase after nerve cutting attributed originally by me to continued nerve action is due to this protection and persistence of the activating substances in fatty materials? An answer to this question might settle not only this particular problem but other related ones in the general field of color change.

One means of attack on this question would be to ascertain by oscillograph methods whether chromatic nerve fibers after having been cut would continue to show action potentials. In making tests of this kind I am under great obligations to Dr. C. L. Prosser, then at the Marine Biological Laboratory, Woods Hole, and to Dr. Hallowell Davis of the Harvard Medical School. Caudal rays from four different fishes were examined for action potentials. Each ray consisted of a flattened, imperfect, cartilaginous tube through the cavity of which extended a bundle of nerve fibers containing chromatic elements. The nerves in some of these rays before tests were made had been activated about 18 hours earlier by cutting, others were freshly cut, and still others were uncut and used as controls. In a group of eight rays four showed some electric activity, physiological or physical, beyond what could be identified as amplifier noises. One of

these was a ray cut the previous night, two were freshly cut, and one was an uncut control. The other four rays three of which had been cut and were in excellent state for possible action potentials showed no trace of such activities. The sensitivity of this apparatus was about 2 microvolts. Strong evidence that the nerve disturbances, where they occurred, were physical and not action potentials is their persistence for at least 3 minutes in a totally excised ray which had been liberally stroked with chloroform. Dr. Davis's conclusion was that no activity that could be identified as physiological was to be observed in any of the rays either activated or quiescent. This inability to observe such activity may be attributed to the large amount of shunting tissue and the insulating sheath of the ray itself for the removal of which no satisfactory technique could be found.

As contrasted with the caudal nerves in the catfish the ophthalmic nerve in this animal is readily accessible for oscillograph tests. When electrodes are applied to this nerve in the dorsal part of the orbit and the snout of the fish is gently stroked a burst of sensory impulses may be observed. Results of this kind were noted in six of nine preparations. Care was taken to distinguish between true responses and the artifacts due to bodily movements of the fishes, the movements of the nerve on the electrodes, and other like disturbances. These observations showed the ready excitability of the ophthalmic nerve. The cutting of this nerve near its central end was followed by an abundant volley of spikes. Attempts were then made in the region external to the ear capsule to cut the autonomic tracts to the exclusion of other nerve bundles and thus to excite only this particular component in the ophthalmic nerve. But no such favorable region could be found, for at all places on the side of the head of the fish these tracts were accompanied by nerve fibers, probably lateral-line components from the vagus complex, and it was impossible to cut one set of fibers without doing the same to the other. Moreover the greatest length of the ophthalmic nerve available for oscillograph tests in the catfish, about 1 centimeter, was too short for satisfactory work. Hence I was forced to abandon this type of test on the ophthalmic nerves as well as on the caudal nerves and I turned to other methods of attack on this question. The most promising of these appeared to be some form of nerve interference whereby the chromatic impulses could be locally and temporarily blocked with the possibility of a subsequent return.

To this end one naturally reverts to anesthetics and other like substances, and for fishes to such agents as magnesium sulfate.

In preparing catfishes for tests with this salt they were enveloped in wet cheese-cloth, fastened sidewise on an inclined pine bench, and provided with a continuous current of

fresh water over their gills. Their caudal fins were then spread out and rendered immobile by being held in place on the top of the wooden bench by push-pins. Two short, parallel cuts were then made through the fin membrane and on opposite sides of a given fin ray. *To these cuts a drop of saturated, aqueous solution of magnesium sulfate was next applied on the assumption that this material would enter the cuts and soon reach the nerves.* A crystal of magnesium sulfate was also placed on the cuts to reinforce the solution. After 40 minutes, to take a particular example, the given ray which had shown no darkening at all, was cut transversely at a point proximal to that at which the salt had been applied. A neighboring ray the sixth from the anesthetized one was also severed at the same time as a control. About 20 minutes later the control ray showed a darkened band, but the anesthetized one was only slightly darkened between the cut and the point at which the magnesium sulfate had been applied. The fact that this ray was fully pale distal to the spot treated with magnesium sulfate showed that this salt had been an effective nerve block. When this fish about an hour later was freed and allowed to swim in a white-walled illuminated tank, the distal part of the anesthetized ray became in the course of an hour or so moderately dark.

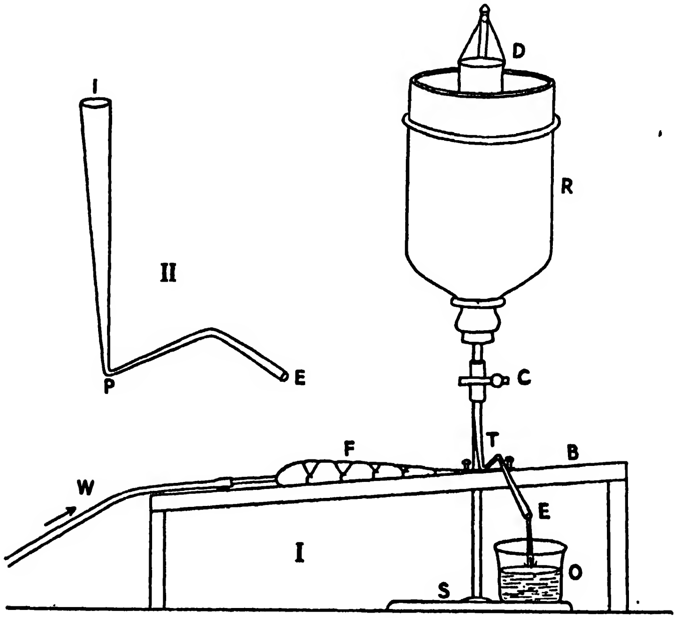
Thus for a period of about an hour the magnesium sulfate blocked some influence which, after the presumable loss of the salt, exerted upon the melanophores the same dispersing effect that cutting the nerve of the control ray had done. Responses of this kind though of constant occurrence were sluggish and far from regular in their time relations. They nevertheless were of such a nature as to make it difficult to understand them except on the basis of a protracted activity of dispersing nerve fibers.

An interesting feature in the tests with magnesium sulfate that should not be lost sight of was the irresponsiveness of anesthetized nerves to cutting. When a nerve was anesthetized as described and after about half an hour was severed in the anesthetized region, no darkening appeared on any part of its length though a cut made distal to the region of anesthetization was soon followed by a darkening of the distal innervated area. This condition points to the inexcitability of the dispersing nerve fibers when under the influence of magnesium sulfate in addition to their inability to conduct impulses, a state that is after all not surprising.

Although the responses of the dispersing nerve fibers when treated with magnesium sulfate are significant, the uncertainty in their timing led me to try other forms of block. Of these I found cold the most effective.

For the application of a cold-block a simple piece of apparatus was devised (Text-fig. 4). This consisted of a glass reservoir (Text-fig. 4, R) made from a bottomless, large bottle held inverted and containing about 1½ liters of 50 per cent alcohol. Into this liquid was lowered a long beaker weighted with lead and containing a supply of dry ice (Text-fig. 4, D). By controlling the amount of ice the temperature of the alcohol mixture could be held at any desired point from −15°C. upwards. The aperture in the bottom of the reservoir, the neck of the bottle, was tightly closed with a rubber stopper

from which a glass tube led to a short rubber tube which carried the blunt cold-point Text-fig. 4, II). This was a piece of glass tubing the lower part of which was of capillary



TEXT-FIG. 4. I. Diagrammatic side view of the apparatus by which a cold-point could be applied continuously to a given ray in the tail of a catfish. II. Enlarged view of the glass cold-point. B, sloping, wooden bench to which the fish F was attached; C, metal screw-clamp for the control of the flow of fluid through the rubber portion of the outlet-tube; D, long, narrow beaker suspended from a rod, weighted with lead, and filled with crushed dry-ice as a means of cooling the liquid in the reservoir R; E, exit for the outflow of cold fluid after it has passed from the reservoir through the glass cold-point; F, living fish wrapped in cloth and bound to the sloping wooden bench by a cord looped through hooks in the top of the bench; I, inlet of the glass cold-point tube: O, overflow beaker to receive the fluid escaping from the cold-point exit; P, elbow in the glass cold-point tube serving as the actual cold-point to be applied to a given ray in the fish's tail; R, glass reservoir filled with fluid, usually 50 per cent alcohol, chilled well below 0°C. by dry-ice, D, and open to discharge through the cold-point tube; S, iron stand with upright iron rod by which the reservoir, R, is held in place by iron rings; T, tail of the fish pinned out immovably on the wooden bench and touched by the cold-point; W, water tube carrying a supply of fresh water to the mouth and gills of the fish for respiration.

fineness and rather sharply bent upon itself at an acute angle. The remainder of the capillary glass tube was turned to one side and directed in such a way as to serve as a convenient outlet (E). The rubber tube connecting the glass tube from the reservoir with that carrying the blunt cold-point was provided with a metal screw-clamp (C) by which the flow of fluid in the apparatus could be controlled. When the cold alcohol mixture was flowing through the tube it ran from the capillary exit at a temperature of

2 or 3° above that in the reservoir. The temperature at the cold-point must have been between these two extremes. With the temperature of the alcohol mixture in the reservoir at about −10°C. that at the capillary exit was some −7°C. and a drop of water put on the cold-point, though exposed to the air, would soon freeze.

Below the cold-point was a small, movable, inclined, pine bench (Text-fig. 4, B) provided with hooks and a cord by which a live catfish (F) enveloped in cheese-cloth could be firmly bound on its side to the top of the bench. By means of glass pushpins the tail of the fish (T) was spread out immovably on the bench and, by shifting the bench slightly any desired part of the fish's tail could be brought into contact with the cold-point. Thus the cold-point, whose diameter was a little over a millimeter, could be applied to any spot on the length of a caudal ray. Fresh water from a rubber tube (W) was led into the mouth of the fish for respiration and flowed out over the gills to escape down the inclined bench. Pale fishes thus attached to the bench could be tested over periods of a number of hours. For some reason not wholly clear they gradually darkened on the bench till their condition was such that they were not very favorable for the inspection of their caudal bands. However fishes that had been bound to the bench 7 to 8 hours were still serviceable and when liberated were fully active and showed no signs of having suffered from their confinement.

When a pale catfish was put in the cold-point apparatus and the fluid was allowed to flow through the point in position on the fish's tail at room temperature, 22°C., no change was observed in the fish except the gradual darkening already noted. When after 15 minutes of flow the ray in contact with the point was cut near the base of the tail a complete, dark, caudal band was formed from the cut to the edge of the tail. The same was true when the temperature of the escaping fluid was about 10°C. When it was 5°C. the caudal band resulting from the cut was almost invariably incomplete in that it could be seen only from the cut to the cold-block and not beyond that point distally. At 0°C. the block was always perfect and no dark band was ever observed distal to the block so long as this outlet temperature was maintained. With the temperature of the fluid at the outlet at 0°C. that in the reservoir was usually 3 to 4° below zero. When the outlet temperature was −10°C. a small cake of ice formed between the cold-point and the fish's tail. This ice spread over a larger area of the tail than the area of the cold-point and as a rule covered about three rays. On releasing a fish thus treated it was found that the spot on the tail immediately below where the ice had been was very pale and that the three rays covered by the ice were dark from the region of the ice almost to the edge of the tail; in other words the very low temperature of the ice cake acted upon the nerves of the rays as cutting them would have done. It was evident from these preliminary tests that the satisfactory temperature for the cold-block was about 0°C., for lower temperature (−10°C.), like nerve severance, induced the formation of dark bands and higher temperatures

(10°C.) were insufficient to restrain the nerve impulses. In operating with the cold-block I therefore subjected the given ray of the fish to a temperature of about 0°C. for some 15 minutes and then proceeded to further experimentation. I have seen nothing in my tests that would lead to the suspicion that this treatment was not wholly satisfactory.

The special experimentation carried out upon catfishes with the cold-block had to do with the interval of time between the cutting of the blocked ray and the extension of the caudal band beyond the block itself after it had been removed and the fish liberated. When a pale catfish was put in the cold-block apparatus and the block was applied at about 0°C. for a quarter of an hour, a severance of the chilled ray proximal to the block was followed by the formation of a dark caudal band from the cut to the block but not beyond it. This was so invariably the case that it may be looked upon without question. If 15 minutes later the block was then removed and the fish allowed to swim in a white-walled aquarium, the caudal band became extended in that it could be seen in about 10 minutes to cover the course of the ray from the cut to near the edge of the tail. It thus came to occupy that part of the ray which was distal to the block and which before the fish was freed was fully pale. It is difficult to understand this extension of the band except by assuming that that nervous activity which when the cut was made induced the formation of the proximal part of the band was also present to excite the production of its distal part.

When tests similar to the one just described were carried out but with longer intervals of time between the cutting of the ray and the removal of the cold-block, the results were precisely like the 15 minute test. The extension of bands distal from the block occurred after intervals of 30 minutes, 1, 2, 3, 5, and even 6½ hours. The longest of these periods was tried twice after which the released fishes showed no signs of exhaustion and developed dark distal bands obviously visible even against the fish's own darkening background.

It has already been shown (Parker, 1940) that in the catfish, caudal bands may be formed, though not at full intensity, in hypophysectomized individuals. It is therefore probable that the extension of these bands in pale fishes of long standing is not necessarily dependent upon the pituitary neurohumor intermedin. To be reasonably secure of this, however, the block test was repeated on hypophysectomized catfishes. Five fishes were deprived of their pituitary glands and were allowed a little less than a week in which to recover. During this interval one died. Of the four remaining fishes two were subjected to tests with the cold-block over periods of about 5 hours each. In both instances on freeing the fish a fairly dark extension

of the caudal band took place showing that this activity was dependent upon nerves. The results from the tests on these two fishes then agree with those on fishes whose pituitary glands were intact and justify the general conclusion that the activity of severed, dispersing nerve-fibers shows a real protraction.

What the means of exciting such severed nerves are is not known. I have elsewhere suggested (Parker, 1934 *a*) that excitation may be due to a chemical activation of the severed ends of the nerves by substances liberated from the adjacent tissues in the cut itself. Such an injury effect chemical in nature might well be compared with the kind of chemical stimulation of the cut ends of nerves as reported on recently by Fessard (1936), Brink and Bronk (1937), and others.

The fact that not only caudal bands but relatively large integumentary areas in such fishes as *Fundulus* and *Ameiurus* may be darkened by nerve cutting, then blanched, and finally again darkened by the recutting of their nerves affords a potent argument against the views that such types of darkening are due to either paralysis (Brücke, 1852) or the exclusion of certain central nervous influences (Zoond and Eyre, 1934). Furthermore revived darkening demonstrates that vasomotor readjustments or other circulatory disturbances suggested as possible causes for integumentary color changes both pale and dark (Hogben, 1924; Lundstrom and Bard, 1932; Young, 1933; Wykes, 1938; Waring, 1938) are not essential to these changes, for the influence on the circulation of cutting a nerve must be fully expended with the first cut and yet the characteristic color changes reappear with the second one. I am therefore of opinion that none of these factors are responsible for the occurrence of melanophore expansion when a given chromatic nerve is severed.

As tests with the cold-block show the kind of activation here seen may persist for at least some hours. Such an extension of nerve activity is repugnant to most neurophysiologists. Accustomed as they are to the momentary activity of a cut nerve as seen in the single twitch of its muscle or in the very brief volley of spikes exhibited in an oscillograph record of its responses they are averse to accepting the idea of an extended period of activity. It must be remembered, however, that Adrian (1930) has shown that the cut nerves in cats and rabbits exhibit fluctuations of electric potential that may last for an hour or more and that when these fluctuations subside, they may be revived by recutting the nerves. Barnes (1930) has also recorded that after the motor nerves of the walking legs in the crab, *Cancer*, are severed nervous discharges of long duration follow. Hoagland (1933) in his study of the lateral-line nerves of fishes has shown that when

these nerves are cut centrally and freed peripherally from their terminal organs, neuromasts, they will exhibit injury discharges which may keep up for from 10 to 15 minutes. More recently Prosser (1934) has demonstrated that the cut nerve to the chela of the crayfish will emit high frequency discharges that ordinarily may be observed for 5 minutes after the preparation has been made.[2] These instances support the idea that after certain nerves have been cut the activity thereby excited may continue for a relatively long period of time. In the case of the dispersing autonomic nerve fibers in *Ameiurus* this period may be as long as $6\frac{1}{2}$ hours.

4. DISCUSSION

In an earlier part of this paper it was pointed out that large skin areas which had been darkened by the cutting of their nerves blanched as completely as caudal bands did. In some of these, such as the cephalic areas in *Fundulus*, the region became pale by lateral encroachment precisely as do the caudal bands in fishes generally. The dark cephalic areas in *Ameiurus*, however, blanched not only by lateral encroachment but also by internal disintegration. Such lateral encroachments have been ascribed to the passage of a blanching neurohumor from the adjacent pale field into the dark area by a cell-to-cell transmission (Parker, 1933) through the lipoid components of the cells (Parker, 1934 *a*, 1935). A neurohumor of this kind would be carried in oily material, a lipohumor, as contrasted with a water-soluble agent or hydrohumor. That this operation is due to an invasion of the dark area by a blanching humor and not to the loss of an opposing humor from the darkened region into the neighboring pale area was shown by Matsushita (1938) who demonstrated a blanching of a dark caudal band on its side next a pale innervated field and the absence of such blanching on its opposite side next a denervated pale field. This explanation of loss of tint by positive invasion applies well to blanching in dark caudal bands or in cephalic areas such as those of *Fundulus* where the whole area disappears by lateral encroachment, but it fails to make clear the way in which internal disintegration of dark areas takes place. Here a pale spot appears inside a dark region and enlarges quite independently of the periphery of that region. This must be due to some other operation than lateral invasion.

[2] Waring (1938) states that O'Shaughnessy and Slome (1935) in their study of experimentally produced traumatic shock, have established the persistent effect of injury currents in nerves. However, the view expressed by these two authors, though in my opinion very probably correct, is advanced by them not as a demonstration, as is implied by Waring, but as a working hypothesis.

What that operation may be is not easy to surmise. It is very probable from the work of the last year or two that in *Ameiurus* the nervous dispersing neurohumor is acetylcholine and the concentrating one adrenalin. The blanching activities that have been discussed in the preceding paragraph must then be due to adrenalin. This substance, as already stated, is moderately stable in the blood and lymph of the catfish where it may remain active for some hours after it has been injected. It partakes of the nature both of a hydrohumor and of a lipohumor, for it may be carried both in water and in oil. In my opinion the two steps in the blanching of such a dark region as a cephalic area in the catfish represent these two conditions of adrenalin. The lateral encroachment on such a dark region is due, I believe, to the action of adrenalin as carried in the lipoids of the adjacent tissues, and the internal disintegration results from the action of this substance as carried in blood and lymph directly under the melanophore layer. Thus lateral encroachment is an invasion by way of the skin and internal disintegration a subdermal invasion. This at least is an explanation of the total blanching of catfish cephalic areas to which I have not been able to find serious objection. According to this view the peripheral blanching of caudal bands and dark areas is due to adrenalin as a lipohumor and the internal blanching of dark areas to the same agent as a hydrohumor. This leads to a suggestive interpretation of the blanching of the pelvic fins in *Ameiurus*. In my study of this phenomenon I have never seen any evidence of a progressive peripheral loss of shade in these fins such as is characteristic of caudal bands and other dark areas. When the pelvic fin blanches it blanches *in toto* and with great uniformity. Such an operation is what would occur if the blanching resulted from the action of adrenalin as a hydrohumor; *i.e.*, adrenalin dissolved in watery lymph. This I believe to be the case and in truth when a dark catfish is injected with the appropriate amount of adrenalin its pelvic fins blanch in precisely this way. This general view of the double action of adrenalin depending upon the way in which it is carried is obviously hypothetical, but as an hypothesis it appears to meet all the requirements of the situation.

From what has been presented in the preceding pages it is evident that the darkening of *Ameiurus* is dependent upon two neurohumors, acetylcholine and intermedin. Although these two substances in general act in the same way on catfish melanophores, they are not precisely similar from an operational standpoint. It is now well established contrary to my original opinion (Parker, 1934 *b*; Osborn, 1938; Parker, 1940), that of these two materials intermedin is the more effective as a chromatic activator.

At full dispersion the pigment of a macromelanophore in *Ameiurus* may cover an area whose diameter is about 145 microns. This degree of dispersion can be accomplished often with intermedin alone, as for instance on completely denervated melanophores in a caudal band, but in a hypophysectomized fish where acetylcholine from nerves is the exclusive dispersing agent only from a quarter to a half of this amount of pigment dispersion is possible. Hence acetylcholine is much less effective than intermedin as a means of darkening catfishes.

Although acetylcholine is second to intermedin as a melanophore activator in this fish it is probably a somewhat earlier agent in initiating darkening. A hypophysectomized palish catfish fully devoid of any functionally significant intermedin and in consequence dependent upon dispersing nerves and their acetylcholine for darkening will begin this process in half an hour after the fish has been put in a black-walled, illuminated aquarium. As contrasted with this a denervated, pale, caudal band in a pale catfish will start darkening only a number of hours or even a day or more after the fish has been placed in a similar aquarium. Thus the nervous agent, acetylcholine, probably really initiates the darkening process in the catfish and is followed and supported only after a considerable interval by the pituitary secretion intermedin. Hence in a second respect the two darkening agents, intermedin and acetylcholine, are noticeably different.

So far as the main question of this research is concerned—the protracted activity of cut chromatic nerves—only an incomplete answer can be given. The evidence herein presented strongly favors the view that dispersing autonomic nerves when cut pass into a state of activity that is greatly protracted in comparison with that of other cut nerves. This extension of activity may last for at least 6½ hours and during that time there is good evidence for a continued discharge of acetylcholine. That this activity may last for days or weeks cannot be asserted though there is no evidence, so far as I am aware, to the contrary. During the life of a dark caudal band which in *Fundulus* may be a day or more, in *Ameiurus* about a week, and in *Parasilurus* some 2 weeks, the earliest part of this period in *Ameiurus* at least is marked by continued nerve activity with the steady discharge of acetylcholine. This state may possibly continue with gradual abatement throughout the rest of the life of the band or it may be gradually replaced by a darkening operation dependent upon excess acetylcholine stored in the neighboring lipoids or it may involve both processes. Of course during the greater part of this whole period the caudal band is accentuated by the presence of the hydrohumor intermedin.

5. SUMMARY

1. When appropriate chromatic nerves are cut caudal bands, cephalic areas, and the pelvic fins of the catfish *Ameiurus* darken. In pale fishes all these areas will sooner or later blanch. By recutting their nerves all such blanched areas will darken again.

2. These observations show that the darkening of caudal bands, areas, and fins on cutting their nerves is not due to paralysis (Brücke), to the obstruction of central influences such as inhibition (Zoond and Eyre), nor to vasomotor disturbances (Hogben), but to activities emanating from the cut itself.

3. The chief agents concerned with the color changes in *Ameiurus* are three: intermedin from the pituitary gland, acetylcholine from the dispersing nerves (cholinergic fibers), and adrenalin from the concentrating nerves (adrenergic fibers). The first two darken the fish; the third blanches it. In darkening the dispersing nerves appear to initiate the process and to be followed and substantially supplemented by intermedin.

4. Caudal bands blanch by lateral invasion, cephalic areas by lateral invasion and internal disintegration, and pelvic fins by a uniform process of general loss of tint equivalent to internal disintegration.

5. Adrenalin may be carried in such an oil as olive oil and may therefore act as a lipohumor; it is soluble in water and hence may act as a hydrohumor. In lateral invasion (caudal bands, cephalic areas) it probably acts as a lipohumor and in internal disintegration (cephalic areas, pelvic fins) it probably plays the part of a hydrohumor.

6. The duration of the activity of dispersing nerves after they had been cut was tested by means of the oscillograph, by anesthetizing blocks, and by cold-blocks. The nerves of *Ameiurus* proved to be unsatisfactory for oscillograph tests. An anesthetizing block, magnesium sulfate, is only partly satisfactory. A cold-block, 0°C., is successful to a limited degree.

7. By means of a cold-block it can be shown that dispersing autonomic nerve fibers in *Ameiurus* can continue in activity for at least 6½ hours. It is not known how much longer they may remain active. So far as the duration of their activity is concerned dispersing nerve fibers in this fish are unlike other types of nerve fibers usually studied.

The work recorded in this paper was done in part at the Harvard Biological Laboratories and in part at the Woods Hole Marine Biological Laboratory and I wish to express here my sincere thanks to the personnel of these two institutions for their kindly cooperation and ready help.

REFERENCES

Abolin, L., 1925, Beeinflussung des Fischfarbwechsels durch Chemikalien. I. Infundin- und Adrenalinwirkung auf die Melano- und Xanthophoren der Elritze (*Phoxinus laevis* Ag.), *Anz. Akad. Wissensch., Math.-naturwissensch. Cl., Wien*, **61**, 170.

Abramowitz, A. A., 1935, Regeneration of chromatophore nerves, *Proc. Nat. Acad. Sc.*, **21**, 137.

Adrian, E. D., 1930, The effects of injury on mammalian nerve fibres, *Proc. Roy. Soc. London, Series B*, **106**, 596.

Barnes, T. C., 1930, Peripheral tonus associated with impulse discharges in crustacean nerve, *J. Physiol.*, **70**, xxiv.

Brink, F., Jr., and Bronk, D. W., 1937, Rhythmic activity of single nerve fibers induced by low calcium, *Proc. Soc. Exp. Biol. and Med.*, **37**, 94.

Brücke, E., 1852, Untersuchungen über den Farbenwechsel des afrikanischen Chamäleons, *Denkschr. Akad. Wissensch. Math.-naturwissensch. Cl.*, Wien, **4**, 179.

Chang, H. C., Hsieh, W. M., and Lu, Y. M., 1939, Light-pituitary reflex and adrenergic-cholinergic sympathetic nerve in a teleost, *Proc. Soc. Exp. Biol. and Med.*, **40**, 455.

Chin, Y., 1939, Does acetylcholine play a part in the mechanism of melanophore expansion? *Proc. Soc. Exp. Biol. and Med.*, **40**, 454.

Dalton, H. C., and Goodrich, H. B., 1937, Chromatophore reactions in the normal and albino paradise fish, *Biol. Bull.*, **73**, 535.

Fessard, A., 1936, L'activité rhythmique des nerfs isoles, Paris.

Foster, K. W., 1937, The blue phase in the color changes of fish with special reference to the role of the guanin deposits in the skin of *Fundulus heteroclitus*, *J. Exp. Zool.*, **77**, 169.

Fries, E. F. B., 1931, Color changes in *Fundulus*, with special consideration of the xanthophores, *J. Exp. Zool.*, **60**, 384.

von Frisch, K., 1911, Beiträge zur Physiologie der Pigmentzellen in der Fischhaut, *Arch. ges. Physiol.*, **138**, 319.

Hoagland, H., 1933, Electrical responses from the lateral-line nerves of fishes. IV. The repetitive discharge, *J. Gen. Physiol.*, **17**, 195.

Hogben, L., 1924, The pigmentary effector system, Edinburgh, Oliver and Boyd.

Kamada, T., 1937, Parker's effect in melanophore reactions of *Macropodus opercularis*, *Proc. Imp. Acad. Tokyo*, **13**, 217.

Kleinholz, L. H., 1935, The melanophore-dispersing principle in the hypophysis of *Fundulus heteroclitus*, *Biol. Bull.*, **69**, 379.

Lundstrom, H. M., and Bard, P., 1932, Hypophysial control of cutaneous pigmentation in an elasmobranch fish, *Biol. Bull.*, **62**, 1.

Matsushita, K., 1938, Studies on the color changes of the catfish, *Parasilurus asotus* (L.), *Sc. Rep. Imp. Univ. Sendai*, 4 ser. Biol. **13**, 171.

Matthews, S. A., 1933, Color changes in *Fundulus* after hypophysectomy, *Biol. Bull.*, **64**, 315.

Mills, S. M., 1932, The double innervation of fish melanophores, *J. Exp. Zool.*, **64**, 231.

Odiorne, J. M., 1937, Morphological color changes in fishes, *J. Exp. Zool.*, **76**, 441.

Osborn, C. M., 1938, The role of the melanophore-dispersing principle of the pituitary in the color changes of the catfish, *J. Exp. Zool.*, **79**, 309.

O'Shaughnessy, L., and Slome, D., 1935, Etiology of traumatic shock, *Brit. J. Surg.*, **22**, 589.

Parker, G. H., 1933, The cellular transmission of neurohumoral substances in melanophore reactions, *Proc. Nat. Acad. Sc.*, **19**, 175.

Parker, G. H., 1934*a*, The prolonged activity of momentarily stimulated nerves, *Proc. Nat. Acad. Sc.*, **20**, 306.

Parker, G. H., 1934*b*, Color changes of the catfish *Ameiurus* in relation to neurohumors, *J. Exp. Zool.*, **69**, 199.

Parker, G. H., 1935, The chromatophoral neurohumors of the dogfish, *J. Gen. Physiol.*, **18**, 837.

Parker, G. H., 1936, The reactions of chromatophores as evidence for neurohumors, in Cold Spring Harbor symposia on quantitative biology, Cold Spring Harbor, Long Island Biological Association, **4**, 358.

Parker, G. H., 1937, Color changes due to erythrophores in the squirrel fish *Holocentrus*, *Proc. Nat. Acad. Sc.*, **23**, 206.

Parker, G. H., 1939, General anesthesia by cooling, *Proc. Soc. Exp. Biol. and Med.*, **42**, 186.

Parker, G. H., 1940, On the neurohumors of the color changes in catfishes and on fats and oils as protective agents for such substances, *Proc. Am. Phil. Soc.*, **83**, 379.

Parker, G. H., and Porter, H., 1933, Regeneration of chromatophore nerves, *J. Exp. Zool.*, **66**, 303.

Pouchet, G., 1876, Des changements de coloration sous l'influence des nerfs, *J. anat. et physiol.*, Paris, **12**, 1–90, 113–165.

Prosser, C. L., 1934, Action potentials in the nervous system of the crayfish. I. Spontaneous impulses, *J. Cell. and Comp. Physiol.*, **4**, 185.

Sand, A., 1935, The comparative physiology of colour response in reptiles and fishes, *Biol. Rev.*, **10**, 361.

Smith, D. C., 1928, The effect of temperature on the melanophores of fishes, *J. Exp. Zool.*, **52**, 183.

Smith, D. C., 1931, The influence of humoral factors upon the melanophores of fishes, especially *Phoxinus*, *Z. vergleich Physiol.*, **15**, 613.

Tomita, G., 1938, The physiology of color changes in fishes. II. The antidromic responses in the melanophore system in the angelfish, *J. Shanghai Sc. Inst.*, sec. IV, **4**, 9.

Vilter, V., 1938, Déterminisme mélano-constricteur de bandes d'assombrissement consécutives aux sections nerveuses dans la nageoire dorsale du Gobius, *Compt. rend. Soc. biol.*, **129**, 1166.

Vilter, V., 1939*a*, Configuration des dermatomes pigmento-moteurs chez les téléostéens et modalités de leur recouvrement réciproque, *Compt. rend. Soc. biol.*, **130**, 388.

Vilter, V., 1939*b*, Evolution des bandes sombres provoquées par la section de nerfs pigmento-moteurs chez les téléostéens. Intervention de la circulation en tant que vecteur des hormones pigmento-motrices, *Compt. rend. Soc. biol.*, **130**, 391.

Waring, H., 1938, Chromatic behavior of elasmobranchs, *Proc. Roy. Soc. London, Series B*, **125**, 264.

Wykes, U., 1938, The control of photo-pigmentary responses in eyeless catfish, *J. Exp. Biol.*, **15**, 363.

Wyman, L. C., 1924, Blood and nerve as controlling agents in the movement of melanophores, *J. Exp. Zool.*, **39**, 73.

Young, J. Z., 1933, The autonomic nervous system of selachians, *Quart. J. Micr. Sc.*, **75**, 571.

Zoond, A., and Bokenham, N. A. H., 1935, Studies in reptilian colour response. II. The rôle of retinal and dermal photoreceptors in the pigmentary activity of the chameleon, *J. Exp. Biol.*, **12**, 39.

Zoond, A., and Eyre, J., 1934, Studies in reptilian colour response. I. The bionomics and physiology of the pigmentary activity of the chameleon, *Phil. Tr. Roy. Soc. London, Series B*, **223**, 27.

EXPLANATION OF PLATES

The photomicrographs on Plates 1 and 2 were taken by Miss Jane Bridgman.

Plate 1

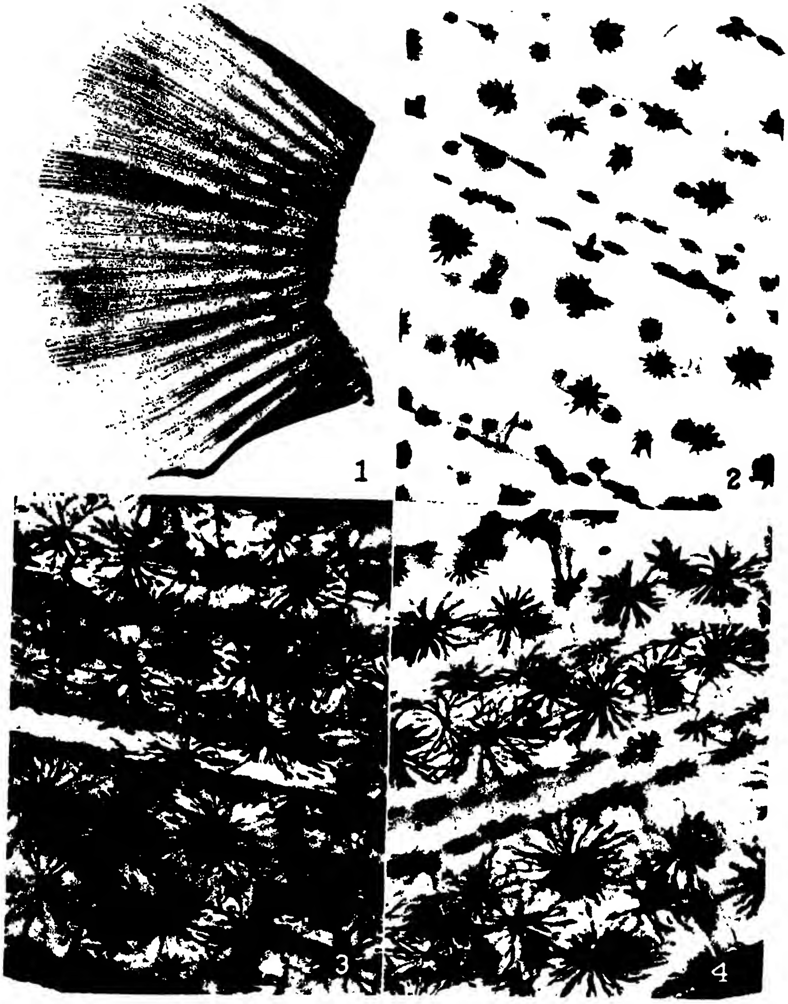
Fig. 1. Tail of a moderately pale killifish, *Fundulus heteroclitus*, showing, above, a newly excited caudal band fully dark and, below, a re-excited caudal band moderately dark. The re-excited band when first cut was as dark as the newly excited one seen in this tail. It was then allowed to blanch fully after which it was re-cut. It then darkened considerably but not completely.

Figs. 2, 3, and 4. Photomicrographs illustrating the conditions of the melanophores in different bands in the tail shown in Fig. 1.

Fig. 2. Melanophores from a pale band with almost fully concentrated pigment.

Fig. 3. Melanophores from the newly cut band; pigment almost fully dispersed.

Fig. 4. Melanophores from the re-excited band; pigment fairly dispersed, but not as much so as in the newly excited band (Fig. 3).



(Parker: Melanophore bands and areas due to nerve cutting)

Plate 2

Fig. 5. Photomicrograph of the pelvic fin of a pale catfish.

Fig. 6. Melanophores from the fin shown in Fig. 5; pigment fully concentrated.

Fig. 7. Pelvic fin of a pale catfish showing near the middle a dark band produced by cutting a ray.

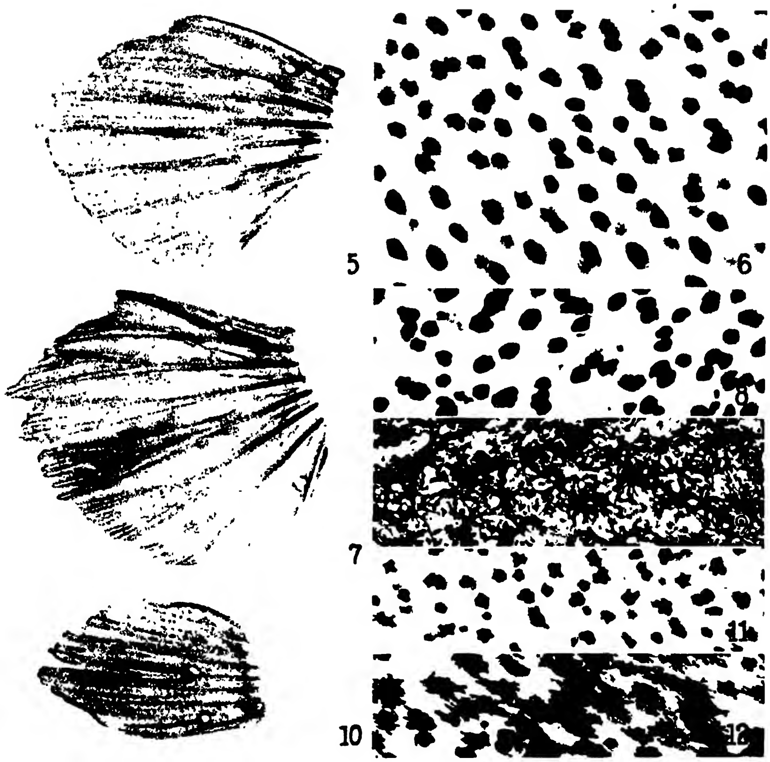
Fig. 8. Melanophores from an uncut ray in the fin shown in Fig. 7; pigment fully concentrated.

Fig. 9. Melanophores from the dark band in Fig. 7; pigment fully dispersed.

Fig. 10. Pelvic fin of a pale catfish fully denervated by having had all the nerves which entered the fin severed in the body of the fish before they reached the fin. After this fin had blanched one ray was cut whereupon this ray darkened somewhat.

Fig. 11. Melanophores from a blanched ray in the fin shown in Fig. 10; pigment almost fully concentrated.

Fig. 12. Melanophores from the cut ray in the fin shown in Fig. 10; pigment partly but not fully dispersed. Compare with Fig. 9.



(Parker: Melanophore bands and areas due to nerve cutting)

THEORY AND MEASUREMENT OF VISUAL MECHANISMS

IV. Critical Intensities for Visual Flicker, Monocular and Binocular

By W. J. CROZIER and ERNST WOLF

(*From the Biological Laboratories, Harvard University, Cambridge*)

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I

On comparing monocular and binocular critical points for visual flicker, within the fovea, it was found by Sherrington (1902, 1904, 1906) that for similar phases of interrupted illumination falling synchronously on each retina there was very little reinforcement, so that the binocular fusion frequency was almost the same as when using one eye (or a little higher). The observations were made at a comparatively high flash intensity, and thus at a high flash frequency, save for some incidental tests. No mention was made of differences in critical flash frequency for the right and left eyes of an observer; such differences are always revealed by systematic tests, in our experience, although at the upper end of the F-log I contour they may not be easy to detect.

Sherrington's data and the conclusions he drew from them, involving other phase relations in the interruptions of light to the two eyes, are frequently referred to in connection with the general problem of "binocular summation;" doubtless others have noted similar findings; but the basic question seems not to have been carefully re-examined. It has never been made entirely clear just in what respect such observations should be expected to reveal evidence of "summation," and in default of a theory of the flicker response contour naïve expectations might very well be obscure. In now making a re-investigation of the relations between monocular and binocular flicker thresholds a chief point has been to establish under optically simple conditions the relation of the binocular flicker response contour to that obtained for each eye taken separately. This has not heretofore been done. It is necessary also to have quantitative information as to the *variability* of such measurements. Relatively complete contours must be obtained for each eye taken separately before any real discussion of "binocular summation" can be attempted. It cannot be

predicted that the relations at low flash frequencies, where the frequency of subjective flicker corresponds to the actual flash rate, will necessarily be the same as at high intensities where it does not; nor can the relations between *subjective brightness-at-fusion, flash intensity,* and *critical frequency be adequately studied without knowledge of the whole curve.*

We have purposely employed for the present experiments a centrally fixated image large enough to provide an excitable extra-foveal area, thus extending the data to the "rod" segment of the duplex performance curve. The flash cycle used for the main observations gave equally long light and dark intervals. In other experiments, dealt with in communications immediately following, we discuss in detail the effects of altering the retinal position of this image, the rôle of the light-time fraction, and the relations of the several response contours to the wave-length composition of the light.

The interrelation between the influences of these variables is of especial significance for the theory of the flicker effect. For our immediate purpose, however, it is important that the same procedure is shown to be successful in analyzing the response contours obtained in these different experiments; the parameters which this analysis reveals may thus be used with confidence for the general comparison of the flicker excitation functions when one eye, the other, and both, are concerned under the same physical conditions. Two practiced observers were used. Certain reproducible differences exist in the visual performance contours of these two individuals. Many additional data are now available for them, and confirm the finding that the same method of analysis applies when the variable of "individual difference" is concerned. The existence of such quantifiable differences of course shows why it is unwise to "average" data from different subjects; indeed this is really forbidden; only by accident could such averaged data exhibit theoretically significant properties.

II

The visual discriminometer already described in detail (Crozier and Holway, 1938–1939 *a*) was employed to form equivalent images in one or the other or both eyes. The right-hand beam in the right-hand arm of the instrument was brought to a focus beyond the mirror P_1 (Crozier and Holway, 1938, 1939 *a*, Fig. 1) in the plane of an accurately cut sector-disc. (This was possible by removal of the brass collar visible in Crozier and Holway, 1938–39 *a*, Fig. 2.) Beyond the sector-disc the beam was collimated and focused on the slit S_1 in the usual way (Crozier and Holway, 1938–39 *a*, Fig. 1). The disc was driven by a controlled-speed motor and gear system; the revolution frequency was determined from millivoltmeter readings of the potential developed by a sensitive magneto geared to the driving shaft (*cf.* Crozier and Wolf, 1939–40 *d*, etc.).

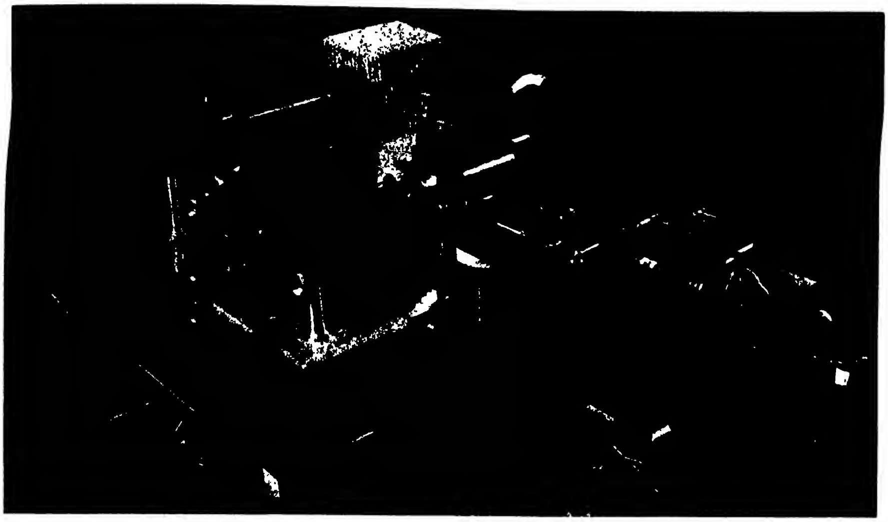

FIG. 1. View of discriminometer with sector wheel in position, driving device, magneto, and lamp ammeter (control desk with millivoltmeter, etc. not shown; observer's cubicle removed); see text.

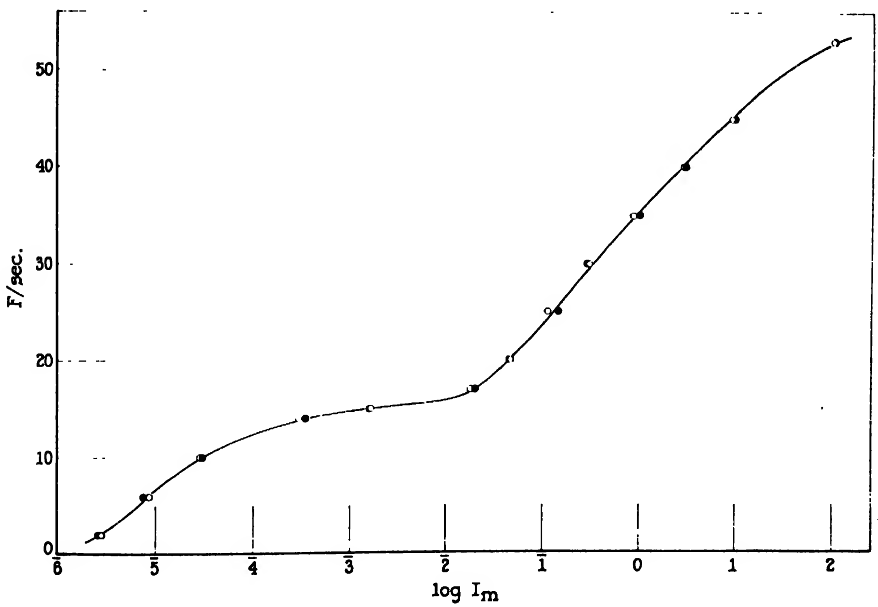

FIG. 2. Comparison of *flicker response* thresholds (flash intensity, I_m), solid circlets; with *fusion* thresholds, open circlets. E. W., left eye, t_L = 0.50, white light (the curve drawn is from: Crozier, Wolf, and Zerrahn-Wolf, 1937-38 *b*).

By using sectors with six or eight openings and a series of interchangeable gears, steady flash frequencies ranging from 2 to about 80 per second could be secured by control of resistances in the motor circuit. The general plan of the apparatus is seen in Fig. 1.

The procedure was to secure observations with the left eye first, then with the right, then with both used simultaneously. The findings were then checked by taking readings with one eye, then with the other, then with both, during single sittings. Each series of measurements was preceded by at least 30 minutes dark adaptation; for work at the lowest (2 to 20) flash frequencies, 45 to 60 minutes. A quite regular procedure of relaxation during the interval of dark adaptation is important in securing regularity of response. Comfort for the observer is insured by the air-conditioned atmosphere of the dark room. A period of several minutes adaptation to each critical fusion intensity level precedes the taking of observations. Each group begins with the lowest flash frequency F desired in that particular set, and the value of F is then fixed at successively higher levels by small steps, with appropriate rest periods during the series. Succeeding groups of determinations are so arranged that there is a partial overlapping of the F ranges; data are taken at eight to ten levels of F in one sitting per day.

It is important for precise observations, taken in such a way as to make possible the study of the variability of readings, that the observer does not control the apparatus in any way. This permits the observer to enjoy relaxed concentration while reducing the possibility of head and eye movements despite the use of the headrest. It also helps to assure reasonable uniformity in the way in which the end-point is approached. After several preliminary trials at each F the approximate value of the critical intensity is known to the person operating the instrument. Then, beginning at a flash intensity about 0.20 log unit below this value, the optical wedge (Crozier and Holway, 1938-39 *a*, Fig. 2) is moved at a nearly constant rate until the observer signals that the intensity for recognition of flicker has been reached. This is repeated until ten readings have been taken. The observer may signal orally, or by means of a foot-switch turning on a small red pilot light.

We have used systematically the determination of the flash intensity I critical for recognition of flicker ("*Flimmern*"), at fixed flash-frequencies F. The curves so produced are of course not *quite* the same as those for the flicker fusion intensities obtained by lowering I at fixed F until fusion is observed; the latter are found to be of the same form but of course tend to be a little below on the intensity scale (Fig. 2); the variability of the critical fusion intensity tends to be a little higher than for the critical flicker intensity.

The discriminometer slit was adjusted to produce on the retina a square image subtending *ca.* 6.13° on a side. In the present experiments the image was centrally fixated. For work at the lowest intensities, a minute red dot produced by a beam in the left-hand arm of the discriminometer (*cf.* Fig. 1; and Crozier and Holway, 1938-39 *a*, Figs. 1 and 2) served as a fixation point in the center of the square. Its intensity could be so adjusted as to make it visible only when focused in the fovea (relaxed accommodation). The cross-section of the beam at the eye-ring is such that its area is less than that of the fully contracted pupil (*cf.* Crozier and Holway, 1938-39 *a*). The square image extending 3°+ vertically and horizontally from the fovea includes that part of the retina known for the particular observers to be of highest intrinsic threshold. Some data for W. J. C. are in Crozier and Holway (1938-39 *b*, 1939-40); the exact form of the curve for threshold intensity as a function of distance from the fovea, of course, depends on the size of the test-patch and on the exposure time, as we know from other work with

these observers; and the excitability of a given retinal area as a whole behaves as a unit in which the observed excitability is determined by the concurrent excitation of spatially contiguous regions (*cf.* Crozier and Holway, 1939–40).

III

Data obtained by the procedure outlined in section II, for E. W. and W. J. C. as observers, are listed in Table I. In considering the properties of such data we have first to deal with the question of intrinsic reliability. This has two separable aspects. These are (1) the reproducibility of the mean values of the critical flicker intensities, and (2) the quantitative properties of the variation among the individual readings. The possible rôle of a particular apparatus and manipulative procedure can be checked, in evaluating the matter of reproducibility, by studying the form of the F-log I contour as already obtained for the same observers by a quite different technic (Crozier, Wolf, and Zerrahn-Wolf, 1937–38 *b*).

We are fortunate in having sets of data on two trained observers of dissimilar ages, obtained with two different pieces of apparatus. We also have indices of the observed dispersions in the homogeneous sets of measurements of which the averages are utilized for the analysis. It should scarcely need emphasis, but apparently still does, that in the absence of measures of scatter there is really no objective criterion of curve-fitting to test a descriptive hypothesis. It is a characteristic of much of the literature of visual theory that this basically significant information is practically never provided. Lacking it, any statement that the curve derived from a specified theory of the underlying mechanism "describes" the observations is, strictly speaking, without meaning unless the parameters of the proposed description can be demonstrated by independent tests to possess the properties the hypothesis implies.

There are two organically different sources of variation in measurements of the kind which concern us here. These are (1) the differences between individuals, and (2) the fluctuating performance of an individual. We are excluding for the moment those variations and differences due to instrumental or manipulative causes; these appear partially in (2), but can influence (1) also, since different individuals may of course react diversely to manipulative differences. It has been a very general practice to seek to increase the significance of measurements of visual excitability by using a large number of observers (again, usually without dispersion indices). Since the several parameters of an excitability function can and do vary quite independently, the quantitative significance of these mean data is in doubt even if the same individuals are employed the same number of

TABLE I

Mean critical flash intensities I_m (millilamberts) for response to visual flicker at different flash frequencies F per sec. Each log I_m is the logarithm for the average of 10 (*ml.*) readings of I_1; the logarithm of the dispersion of these 10 is given as log P.E.$_{1I_1}$. For each of the two observers data are given as derived from the use of the *left* eye, of the *right* eye, and of *both* eyes under conditions of binocular fusion, in independent series of measurements.

F	Obs.: W. J. C.						Obs.: E. W.					
	Left eye		Right eye		Binocular		Left eye		Right eye		Binocular	
	log I_m	log P.E.$_{1I_1}$	log I_m	log P.E.$_{1I_1}$	log I_m	log P.E.$_{1I_1}$	log I_m	log P.E.$_{1I_1}$	log I_m	log P.E.$_{1I_1}$	log I_m	log P.E.$_{1I_1}$
2	$\bar{4}.0584$	$\bar{6}.9046$	$\bar{4}.0496$	$\bar{6}.5752$	$\bar{4}.0311$	$\bar{6}.2741$	$\bar{4}.2524$	$\bar{5}.4634$	$\bar{4}.1764$	$\bar{5}.2914$	$\bar{4}.2813$	$\bar{5}.0077$
	$\bar{4}.0603$	$\bar{6}.7968$										
	$\bar{4}.2418$	$\bar{6}.9303$										
3	$\bar{4}.0839$	$\bar{6}.8647$	$\bar{4}.0518$	$\bar{6}.5475$	$\bar{4}.0842$	$\bar{6}.5262$						
4	$\bar{4}.2127$	$\bar{6}.9748$	$\bar{4}.1035$	$\bar{6}.8150$	$\bar{4}.1810$	$\bar{6}.6284$	$\bar{4}.4730$	$\bar{5}.6462$	$\bar{4}.3673$	$\bar{5}.3822$	$\bar{4}.4786$	$\bar{5}.2315$
5	$\bar{4}.2052$	$\bar{6}.9648$	$\bar{4}.1746$	$\bar{6}.7148$	$\bar{4}.1928$	$\bar{6}.8352$						
6	$\bar{4}.3744$	$\bar{6}.9267$	$\bar{4}.2490$	$\bar{6}.6201$	$\bar{4}.3007$	$\bar{6}.8720$	$\bar{4}.5932$	$\bar{5}.5157$	$\bar{4}.5320$	$\bar{5}.2856$	$\bar{4}.6399$	$\bar{5}.4707$
7	$\bar{4}.3738$	$\bar{6}.9344$	$\bar{4}.2761$	$\bar{6}.9567$	$\bar{4}.3522$	$\bar{6}.7641$						
8	$\bar{4}.4624$	$\bar{6}.8956$	$\bar{4}.3795$	$\bar{6}.8879$	$\bar{4}.4163$	$\bar{6}.8502$	$\bar{4}.8530$	$\bar{5}.8060$	$\bar{4}.7534$	$\bar{5}.6037$	$\bar{4}.8503$	$\bar{5}.6158$
9	$\bar{4}.5231$	$\bar{5}.0759$	$\bar{4}.4469$	$\bar{6}.8818$	$\bar{4}.4771$	$\bar{6}.9956$						
10	$\bar{4}.6457$	$\bar{5}.4819$	$\bar{4}.5668$	$\bar{5}.1970$	$\bar{4}.5990$	$\bar{5}.1587$	$\bar{3}.1440$	$\bar{4}.1436$	$\bar{4}.8905$	$\bar{5}.7810$	$\bar{3}.0561$	$\bar{5}.9438$
							$\bar{3}.2620$	$\bar{4}.3884$	$\bar{4}.9057$	$\bar{5}.7909$	$\bar{3}.0090$	$\bar{4}.0410$
11			$\bar{4}.6487$	$\bar{5}.1202$	$\bar{4}.6736$	$\bar{5}.0469$						
12	$\bar{4}.9784$	$\bar{5}.8206$	$\bar{4}.6798$	$\bar{5}.0542$	$\bar{4}.7570$	$\bar{5}.3147$	$\bar{3}.3815$	$\bar{4}.3038$	$\bar{3}.2514$	$\bar{5}.9885$	$\bar{3}.2887$	$\bar{4}.0755$
13	$\bar{3}.0107$	$\bar{5}.8420$	$\bar{4}.8074$	$\bar{5}.2055$	$\bar{4}.9055$	$\bar{5}.5236$						
14	$\bar{3}.0599$	$\bar{5}.7824$	$\bar{3}.0149$	$\bar{5}.8034$	$\bar{3}.0744$	$\bar{5}.4927$	$\bar{3}.6546$	$\bar{4}.7775$	$\bar{3}.5993$	$\bar{4}.5696$	$\bar{3}.5722$	$\bar{4}.4601$
					$\bar{3}.0157$	$\bar{5}.4153$						
15	$\bar{3}.1492$	$\bar{5}.7287$	$\bar{4}.9619$	$\bar{5}.5509$	$\bar{3}.0867$	$\bar{5}.5689$						
16	$\bar{3}.4352$	$\bar{4}.2140$	$\bar{3}.1587$	$\bar{5}.7862$	$\bar{3}.2521$	$\bar{5}.7287$	$\bar{3}.8871$	$\bar{4}.8049$	$\bar{3}.8432$	$\bar{4}.7027$	$\bar{3}.8842$	$\bar{4}.7194$
17	$\bar{3}.4757$	$\bar{4}.0330$	$\bar{3}.3187$	$\bar{5}.7900$	$\bar{3}.4103$	$\bar{5}.7011$						
					$\bar{3}.3932$	$\bar{5}.9865$						
18	$\bar{2}.2964$	$\bar{3}.0618$	$\bar{3}.5597$	$\bar{5}.9312$	$\bar{3}.6333$	$\bar{4}.0972$	$\bar{2}.3768$	$\bar{3}.2667$	$\bar{2}.1741$	$\bar{3}.2030$	$\bar{2}.2325$	$\bar{3}.0039$
19	$\bar{2}.0195$	$\bar{4}.8126$	$\bar{3}.8122$	$\bar{4}.3874$	$\bar{3}.9256$	$\bar{4}.3398$	$\bar{2}.4698$	$\bar{3}.6761$	$\bar{2}.4115$	$\bar{3}.5514$		

20	$\bar{2}.8793$	$\bar{3}.8793$	$\bar{2}.7852$	$\bar{3}.6351$	$\bar{2}.6151$	$\bar{3}.2036$	$\bar{2}.6682$	$\bar{3}.8412$	$\bar{2}.5843$	$\bar{3}.6153$	$\bar{2}.5322$	$\bar{3}.3766$
			$\bar{2}.4288$	$\bar{4}.9821$	$\bar{2}.6305$	$\bar{3}.1617$	$\bar{2}.6694$	$\bar{3}.5319$	$\bar{2}.6632$	$\bar{3}.5060$	$\bar{2}.5456$	$\bar{3}.2519$
	$\bar{2}.2880$	$\bar{4}.8963$	$\bar{2}.4130$	$\bar{3}.1129$								
	$\bar{2}.5331$	$\bar{3}.2475$										
	$\bar{2}.3312$	$\bar{4}.9407$										
	$\bar{2}.5945$	$\bar{3}.1813$										
22			$\bar{2}.9550$	$\bar{3}.5144$	$\bar{1}.0000$	$\bar{3}.3440$	$\bar{2}.8554$	$\bar{2}.0145$				
22.5									$\bar{2}.8760$	$\bar{3}.7823$		
25	$\bar{1}.2849$	$\bar{3}.9212$	$\bar{1}.1687$	$\bar{3}.7999$	$\bar{1}.2572$	$\bar{3}.9141$	$\bar{1}.0745$	$\bar{2}.2936$	$\bar{1}.0374$	$\bar{2}.1928$	$\bar{1}.0179$	$\bar{3}.5344$
	$\bar{1}.4784$	$\bar{2}.2143$										
27							$\bar{1}.2315$	$\bar{2}.3369$				
28	$\bar{1}.4924$	$\bar{2}.0656$	$\bar{1}.3500$	$\bar{3}.7773$	$\bar{1}.4649$	$\bar{3}.8132$						
30	$\bar{1}.6724$	$\bar{2}.4577$	$\bar{1}.5454$	$\bar{2}.5185$	$\bar{1}.5710$	$\bar{2}.0228$	$\bar{1}.4658$	$\bar{2}.5732$	$\bar{1}.3815$	$\bar{2}.6021$	$\bar{1}.3218$	$\bar{2}.2041$
33	$\bar{1}.8452$	$\bar{2}.4768$	$\bar{1}.7295$	$\bar{2}.3420$	$\bar{1}.7481$	$\bar{2}.3168$	$\bar{1}.7201$	$\bar{2}.6812$				
							$\bar{1}.7422$	$\bar{2}.7486$				
35	0.0149	$\bar{2}.6809$	$\bar{1}.9179$	$\bar{2}.4404$	$\bar{1}.9258$	$\bar{2}.0302$	0.0212	$\bar{1}.1248$	$\bar{1}.7204$	$\bar{2}.5527$	$\bar{1}.6841$	$\bar{2}.4357$
38	0.2117	$\bar{2}.8481$	0.1319	$\bar{2}.6733$	0.1581	$\bar{2}.6183$	0.1480	$\bar{1}.3259$	0.0111	$\bar{1}.0052$		
							0.1909	$\bar{1}.0888$				
40	0.4528	$\bar{2}.9929$	0.3204	$\bar{2}.6427$	0.3404	$\bar{2}.7925$	0.3583	$\bar{1}.0899$	0.1973	$\bar{1}.3412$	0.1446	$\bar{1}.1086$
							0.3944	$\bar{1}.1605$				
43	0.6432	$\bar{1}.1119$	0.5671	$\bar{2}.9893$	0.5938	$\bar{1}.2304$	0.6632	$\bar{1}.4962$	0.4890	$\bar{1}.2425$	0.4419	$\bar{1}.1399$
45	0.9806	$\bar{1}.5575$	0.8231	$\bar{1}.4787$	0.9176	$\bar{1}.3282$			0.7980	0.0535	0.8003	$\bar{1}.5594$
46							1.0224	0.1277				
47	1.2629	$\bar{1}.8208$			1.1351	$\bar{1}.6050$					0.9770	$\bar{1}.7377$
48	1.4505	$\bar{1}.8720$	1.1984	$\bar{1}.8364$	1.3298	$\bar{1}.5563$	1.2492	0.1723	1.1685	0.0298		
							1.1884	0.4379				
49	1.4904	0.0212			1.4685	0.0000						
50	1.7294	0.5087	1.5092	0.0570	1.6530	0.0318	1.4616	0.2960	1.4887	0.3981	1.4079	0.3780
											1.4273	0.3100
51	1.7707	0.1986	1.6847	0.2215	1.7401	0.1732						
52	1.8497	0.3060			1.9836	0.3520	1.7212	0.6075	2.0398	0.9914		
					1.9936	0.6574						
53	2.2472	0.7178	2.1937	0.7273	2.1844	0.8397					1.9756	0.9250
54	2.9214	1.5664			2.4973	1.0630	2.1665	1.2538	2.6957	1.8585		
55	3.0183	1.9996	3.0785	1.8295	2.6766	1.1688	2.6406	1.6724			2.5001	1.5671
					2.6149	1.3638						

times, with the same procedure, at each of the points on the excitability contour. All that can then be said, really, is that such data partially express the experimenter's conception of the "normal state." However great the value of this might conceivably be, it is after all not the primary concern in researches supposedly designed to elucidate the nature of visual excitability. Cases do arise in which a group of individual organisms can be shown by analysis to be effectively a homogeneous group as regards excitability (*cf.* Crozier, 1935; Crozier, Wolf, and Zerrahn-Wolf, 1936–37 *a*, *b*, 1937–38 *a*, 1938–39 *a*, etc.). This cannot in general be done for groups of human observers. The only basis for sense in this matter is the use of adequately numerous data on single observers, with the indices of variation permitting use of objective criteria as to whether a sufficient degree of homogeneity in the measurements does exist.

Just how serious differences can be between observers surely "normal" is sufficiently exemplified in Fig. 3. "Averages" from such curves would be meaningless. The contrasts apparent in Fig. 3 are of an order quite different from the fluctuations of successive determinations with one observer. The properties of these fluctuations are quantitatively definable in a simple way, and have a usable significance.

For each of the observers in the present experiment we already have available (Crozier, Wolf, and Zerrahn-Wolf, 1937–38 *b*) flicker response contours with the left eye, using a quite different apparatus. The centrally fixated image was square, and larger (14.3° on a side) than in the present case. The "rod" component of the duplex curve is therefore more prominent. For the older observer (W. J. C.) there is in the "cone" branch of the curve (Fig. 4) no real difference between the new data (Table I) and those already published. For E. W., younger and more likely to be at an age for more rapid visual change, a real although slight difference is evident. For the rod (*i.e.*, extra-foveal) part of the response contour we have a rather striking difference between E. W. and W. J. C.; for the latter the rod segment runs to lower intensities. Qualitatively, this difference is likewise seen in the older data, where the larger test-area is responsible for a larger rod segment with each observer. The important point is that the cone data are shown to be essentially reproducible, quantitatively (with due respect to the age factor), and likewise the qualitative character of the relative rod contribution, independently of the apparatus used and over a period of several years.

Referring once more to Fig. 3, a further comment must be made concerning homogeneity, averaging, and the estimation of comparative excitabilities. It is obvious that when the homologous performance contours for

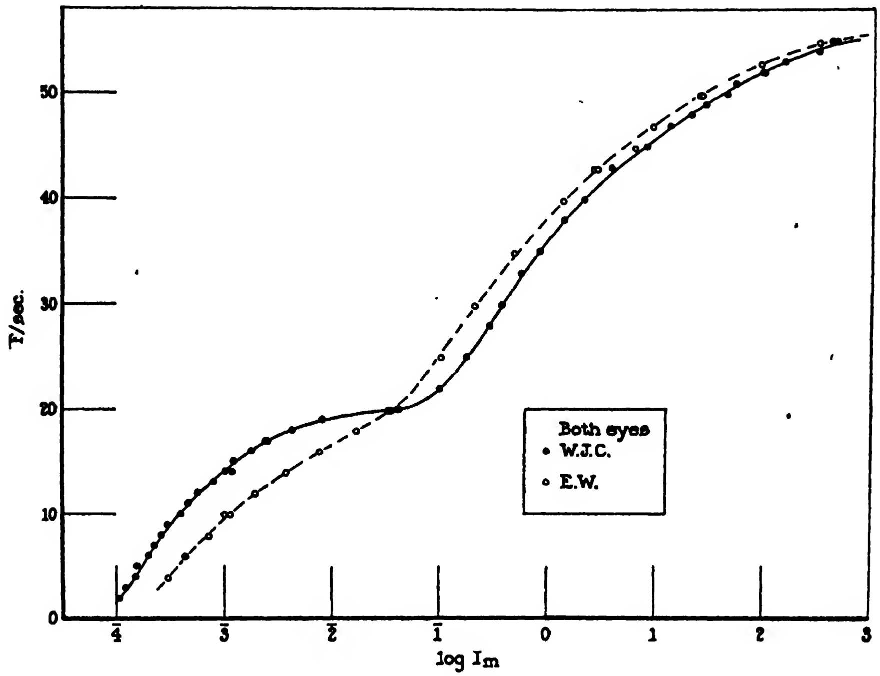

FIG. 3. Comparison of (binocular) flicker response contours for two observers, under the same conditions of observation (Table I).

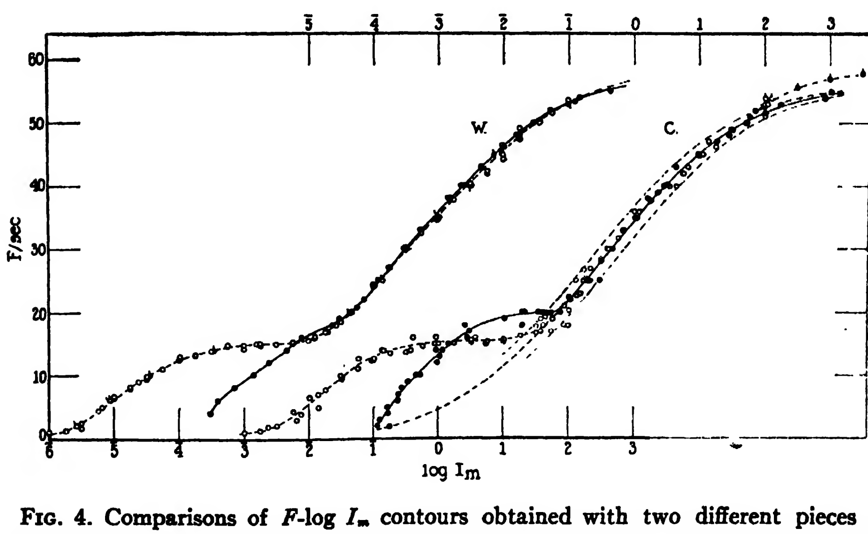

FIG. 4. Comparisons of F-log I_m contours obtained with two different pieces of apparatus, at an interval of 2.5 years, for two observers. Open circlets, data from Crozier, Wolf, and Zerrahn-Wolf, 1937–38 *b*; solid dots, data of the present paper (Table I). The older data with a 14.3° square field, the newer field 6.13° on a side; t_L = 0.50, white light. See text.

two individuals cross one another—a situation not at all unusual—the only possibly valid method of comparing their performance capacities involves and depends upon the theory of the contour as a whole (*cf.* Crozier and Pincus, 1929–30; Crozier and Wolf, 1938–39, 1939–40 *a*; etc.). It is also clear that no simple transformation of the curves, such for example as equating the maximal or the median excitabilities, will bring the two curves into even approximate uniformity. In a similar way the comparison of excitabilities using the right and left eyes of one individual is also faced with the necessity of using complete performance contours.

IV

The data of the flicker response threshold for the comparison of results using one eye, the other, and both, with white light (Table I), are plotted in Figs. 5 and 6. For each of the observers it happens that in ordinary use the *left* is definitely the dominant eye. Yet, as we know from much other data (especially for W. J. C.), "absolute" visual thresholds under given conditions are lower for the *right* eye. They also tend to be lower for E. W. than for W. J. C., although at given levels of I_1 the values of ΔI are lower for W. J. C. As already shown in Fig. 3, the F-log I contours for the two observers cross. For each of them the log I_m values are persistently *lower* for the right eye than for the left, while at the fusion frequency and intensity the field presented to the *right* eye (R) is subjectively brighter than that seen by the *left* (L), although the intensity is lower. These effects are in general accentuated when colored lights are used. They cannot be accounted for by imperfections of the binocular head of the discriminometer or its matched oculars. This is easily checked by repeating the tests with one eye through the ordinarily opposite limb of the head.

The difference between R and L is systematic and statistically significant, but it is not constant. It is a matter of the form of the entire F-log I_m curve, just as in the comparison illustrated in Fig. 3. For E. W., the R and L curves actually cross near the upper end. We shall consider the form of the R and L curves in some detail before taking up the binocular (B) curves, since the intercomparisons depend upon the use of assignable values of the parameters. The analysis of the variation data is then separately considered, in section VI, since it has an important bearing, of a kind apparently not hitherto suspected, upon the decisions which can be made concerning binocular summation.

It has been shown for an adequate variety of organisms that when there is evidenced the visual activity of a simplex performance system the

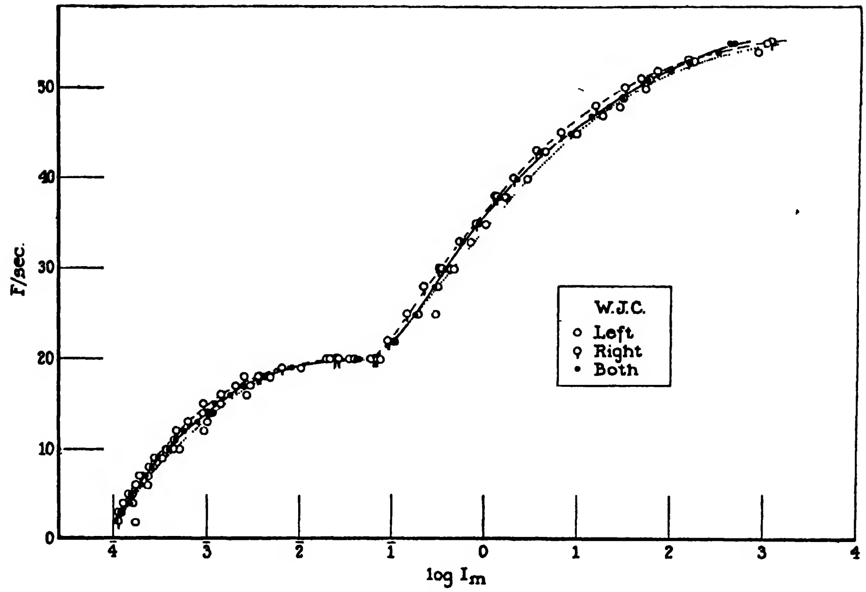

FIG. 5. F-log I_m contours, white light, t_L = 0.50, for W. J. C. (Table I); left eye, right eye, and with binocular fusion. (At F = 30 and 38 extra determinations not entered in Table I have been plotted.)

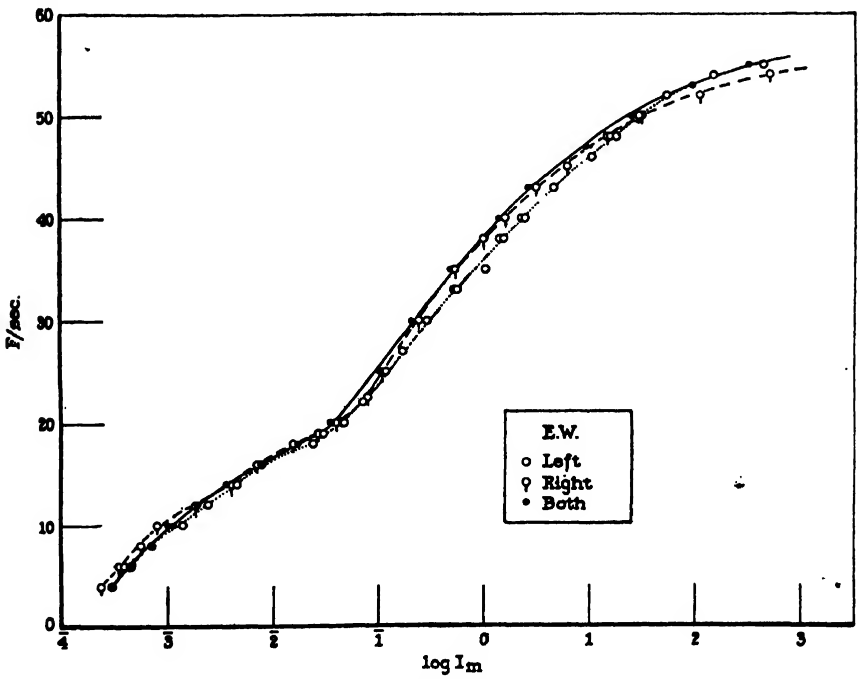

FIG. 6. As in Fig. 5, but for observer E. W.

relation between F and log I is accurately described over its whole extent by a normal probability integral (Crozier, Wolf and Zerrahn-Wolf, 1938–39 *a*, *b*, *c*; Crozier and Wolf, 1940–41 *a*, *b*). The external form of the optic surface in the typical arthropod eye distorts this curve, in a predictable way (Crozier, Wolf, and Zerrahn-Wolf, 1936–37 *b*, 1937–38 *c*; Crozier and Wolf, 1939, 1939–40 *a*). The use of this particular descriptive function rests only in part upon its obvious success in adhering to the data; a more decisive justification is found in the nonspecific rules for the modification of its parameters when the temperature of the organism, the light-time fraction in the flash cycle, and certain other variables, are systematically altered (Crozier, Wolf, and Zerrahn-Wolf, 1937–38 *d*, 1938–39 *a*; Crozier and Wolf, 1939–40 *d*, etc.).

By the use of this function a separation has been made of the two groups of neural effects apparent in the duplex performance curve typically obtained with vertebrates (*e.g.*, Crozier and Wolf, 1938–39, 1939–40 *b*). This procedure has also been applied to flicker response data for man (Crozier, 1937; Crozier, Wolf, and Zerrahn-Wolf, 1937–38 *b*). In the case of various fishes studied, the analysis provides descriptions of groups of neural effects so widely separated on the log I axis that no interference is detectable between the rising curves of the two populations (Crozier and Wolf, 1938–39, etc.). This is proved by the fact that the shape of the curve gives the same form constant when the curve as a whole has been shifted by altering the temperature or the light-time fraction in the flash cycle, even when the two portions of the duplex contour are not affected to the same extent. The important point in this connection has to do with the invariance of the shape constant for the low intensity segment of the curve. The behavior of this constant in cross-breeding experiments with fishes is—like that of the shape constant for the upper segment—entirely consistent with the idea that for these cases the shape constant in question is an organic invariant (*cf.* Crozier and Wolf, 1938–39, 1939–40 *b*).

The situation is significantly different for some vertebrates in which the overlapping of the low and high intensity populations of effects is more complete (as seen with man, frog, *Triturus*, *Fundulus*: Crozier, Wolf, and Zerrahn-Wolf, 1937–38 *b*; Crozier and Wolf, 1939–40 *c*, *e*, *f*). In these latter cases there is reason to believe (*cf.* particularly the following paper: Crozier and Wolf, 1940–41 *c*) that the actual form of the rising low intensity segment of the F-log I curve is the outcome of neural integrations resulting in the partial suppression of effects in the low intensity group. The evidence for this is chiefly derived from experiments in which both wave-length of light and the light-time fraction are varied, and in different

retinal positions. The general properties detected in these experiments are kept in mind in the subsequent analysis.

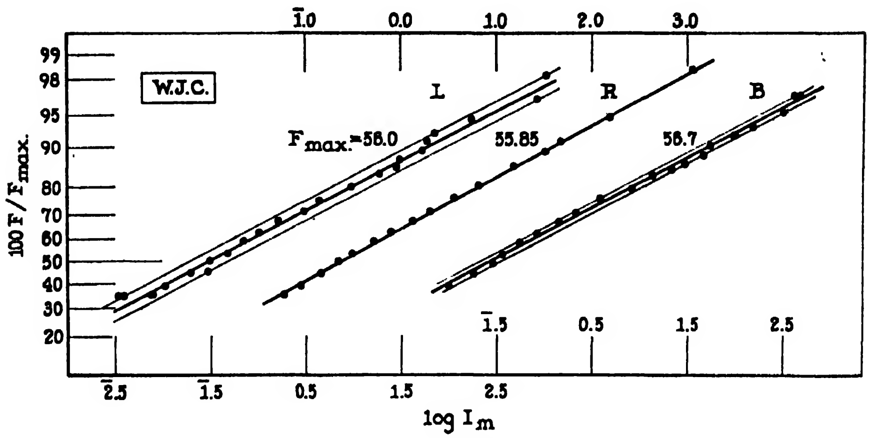

FIG. 7. The upper segments of the graphs of Fig. 5 transferred to a probability grid, and separated laterally for clearness. The asymptotic values of $F_{max.}$ are indicated.

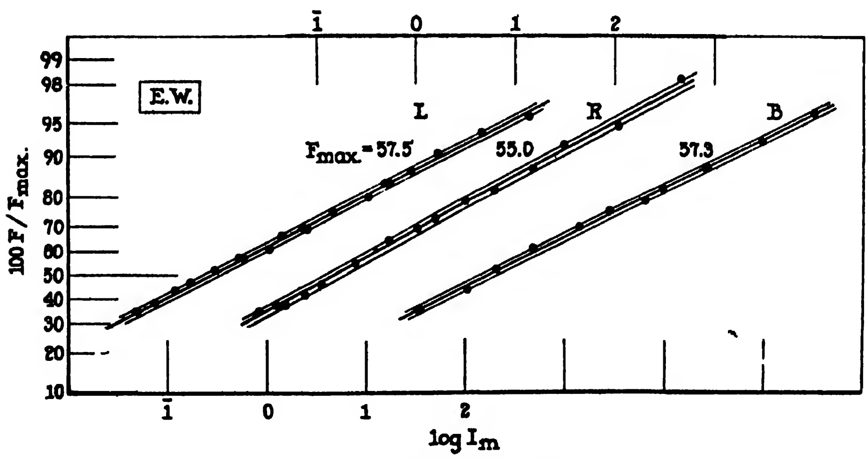

FIG. 8. As in Fig. 7, for the data of Fig. 6.

In these discussions we have been well aware of the traditional assumption that the low intensity segment of such duplex curves of vertebrate visual performance is due to the activation of retinal rods, the high intensity segment to the activation of retinal cones. This assumption has tended to take the form (*cf.* Hecht, 1937) that the quantitative properties of the two segments directly represent and measure respective quantitative properties of the rods and cones as excitable units. For this proposition, in this ex-

treme form, no real basis whatever can be found. As a matter of convenience in reference, however, we have used the designations "rod" and "cone" for the two sections of the duplex contour; the quotation marks signify that we do not take the *form* of the curves to represent in any direct way the characteristics of the retinal sensory cells.

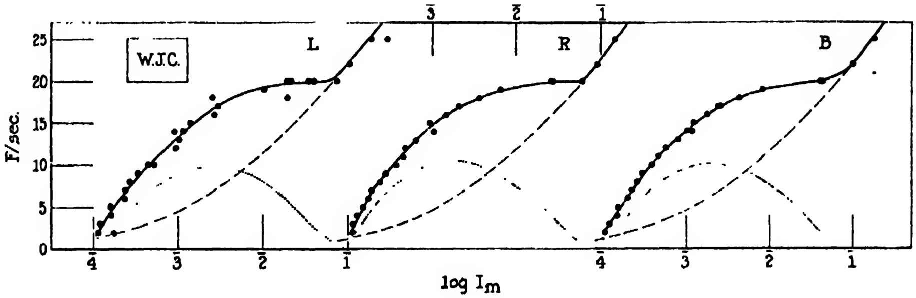

FIG. 9. The extrapolation of the curves in Fig. 7 and the difference curves obtained by subtraction from the data of Fig. 5.

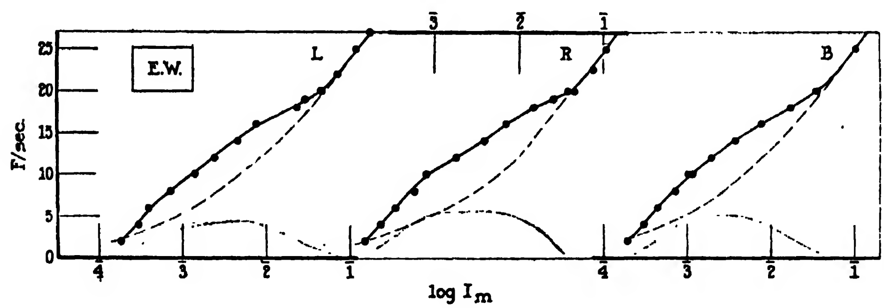

FIG. 10. The lower segment of the data for E. W. (Fig. 6) analyzed as in Fig. 9, on the basis of Fig. 8.

The cone portions of the data of Figs. 5 and 6 are shown on a normal probability grid in Figs. 7 and 8. Confining attention for the moment to the *R* and *L* measurements, it is seen that in each case the value of $F_{max.}$ required for an adequate fit is definitely higher for *L*. With W. J. C. the slope constant ($\sigma'_{\log I}$) is a little higher and the abscissa of inflection τ' a little lower (0.15 log unit) for *R*. With E. W. the situation is essentially the same. The criteria for an adequate fit are rectilinearity on the probability grid and the parallel margins of scatter of the points. The basis for the use of the latter criterion is indicated in section VI.

Following the process already used in the study of many other cases already referred to, the probability integrals of Figs. 7 and 8 have been

extrapolated toward $F = 0$ (Figs. 9 and 10); by ordinate differences the rod contributions shown as dotted lines are then obtained. The rising

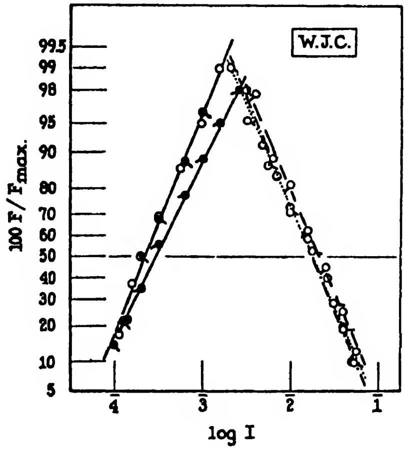

FIG. 11. The dissected-out "rod" curves of Fig. 9 (W. J. C.) transferred to a probability grid: for R, dots with right-side tag; for L with tag on the left; circlets, B. The values used for $F_{max.}$ are: R, 10.5; L, 10.0; B, 10.1. The points plotted are read from the dotted curves in Fig. 9.

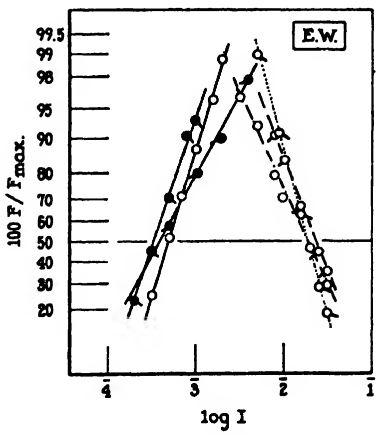

FIG. 12. The isolated "rod" curves for E. W. (Fig. 10) on a probability grid, as in Fig. 11. The values used for $F_{max.}$ are: R, 5.5; L, 4.5; B, 5.2.

and the falling branches of these dotted curves also exhibit rectilinearity upon a probability grid (Figs. 11 and 12). The raw rod data (Figs. 5 and 6) do not. For W. J. C. and E. W. the corrected rod $F_{max.}$ is definitely higher for R than for L, the slope constant is higher, and τ' less. The

declining curves do not differ much in $\sigma'_{\log I}$ or in τ'; the evidence for considering the form of these declining curves as resulting from inhibition of rod effects by cone effects is considered elsewhere (Crozier and Wolf, 1938–39; 1940–41 *c*, *d*).

V

Figs. 5 to 12 inclusive also contain the measurements for the mean binocular (*B*) flicker thresholds. We shall consider the *B* parameters in relation to those for *R* and *L* before dealing with the variation data (section VI). Both are necessary for the theoretical analysis, and they supplement one another in perhaps unexpected ways. It is necessary also to record some subjective effects concerning brightness.

As shown in Figs. 5 and 6, the mean *B* thresholds for E. W. adhere rather closely to, or are a little below, those obtained for the eye (*R*) with lower threshold, over most of the *F* range; at the two ends of the range, however, they agree rather well with those for the other eye (*L*). For W. J. C. the *B* data fall in between the *R* and *L* measurements except at the very top; it cannot be said that they are the arithmetic mean or the geometric mean of the *R* and *L* figures, however; as with E. W., the *form* of the *B* curve is not the same as that for either eye used alone. Obviously, no statement comparing simply the effects of monocular with binocular flicker excitation can be unambiguous in the absence of detailed information over the entire explorable range. While it is true that conditions can be found (as near the crossing point of the *R* and *L* curves for E. W., Fig. 6) such that a very small difference exists between *B* and *R*, *L*, this is not the characteristic state of affairs. Although it is true, as Sherrington (1904) described, that the *B vs. R*, *L* difference is slight, it is nevertheless real and systematic.

The *B* contours are analyzed into their constituent branches in Figs. 8 to 12. The probability integral formulation is just as efficient as for the monocular data. It emerges that with W. J. C. the *B* cone value of $F_{max.}$ is definitely higher than for *R* or *L*, the slope constant $\sigma'_{\log I}$ is intermediate, and the abscissa of inflection τ' is exactly intermediate. With E. W. on the other hand, the *B* $F_{max.}$ is not certainly different from that for *L*, $\sigma'_{\log I}$ is less than for either *R* or *L*, but τ' is again intermediate. It is to be added that when the light-time fraction is systematically modified, and for a given wave-length composition, in a given retinal region, the value of the $\sigma'_{\log I}$ for the rod (rising) curve as analyzed out is found to be invariant despite the unequal shifts of the rod and cone contributions; the significance of this for the statistical basis of the observed functions is

mentioned elsewhere (Crozier and Wolf, 1940–41 *c, d, e*). Thus the statement which can be made for these two observers is that log I_m for the inflection of the binocular cone F-log I contour is rather precisely intermediate between that for the right and the left eye monocular observations. It is exhibited in these results that there is no necessary correlation between the changes in the three parameters of the probability summation,—as is also clearly demonstrated by the various experiments in which the curve is modified experimentally (*cf.* Crozier and Wolf, 1940–41, *c, d, e*).

The "B" rod curves obtained in Figs. 9 and 10 are analyzed in Figs. 11 and 12. For the W. J. C. curve the B $F_{max.}$ is about that for L, but the rising curve does not differ otherwise from that with R. The E. W. rising B curve is also quite like the R. (The general nature of the *declining* branches has been referred to already.) It is to be mentioned that in our earlier experiment, with a larger test area centrally fixated, the rod curve is one with a higher $F_{max.}$, a lower τ', and a much greater $\sigma'_{\log I}$ (*cf.* Crozier, Wolf, and Zerrahn-Wolf, 1937–38 *b*).

The general conclusion to which Sherrington (1904) came from his experiments with symmetrical binocular flicker and brightness was that the binocular perception results from the combination of fully "elaborated uniocular sensations," and is the product of "already elaborated sensations contemporaneously proceeding." With this we agree. In Sherrington's observations he found reason to doubt whether the well known slight excess of binocular *brightness* over that of the uniocular components was really to be explained as due to summation of the intensities of effects at the corresponding points of the two retinas, and that, most often, the binocular brightness was not perceptibly different from that of either of its co-equal uniocular components. Under the conditions of the present observations there is no possible complication due to the consensual pupillary reflex, or to changes of accommodation, and care was always taken to continue comparisons until no differential effect of adaptation could play a part. Yet we find that the L and R subjective brightnesses just at fusion are not equal, that for R being the greater in these tests, and that the B subjective brightness at critical fusion is always *above* that for either eye taken alone—yet the R critical intensity is characteristically lower than the L while the B critical intensity over most of the range either agrees with that for R or lies between the R and L.

Obviously, the relation between F and log I_m is not determined by the subjective brightness alone. This conclusion is well reinforced by the consideration of data in which wave-length of light and light-time fraction in the flash cycle are involved as variables (Crozier and Wolf, 1940–41 *c, d*).

With the apparatus we have used it is also possible to show that not even binocular *fusion* of uniocular images is required for the effects discussed. By separation of the ocular barrels of the discriminometer head (Fig. 1; and *cf.* Crozier and Holway, 1938–39 *a*) a little beyond the distance for

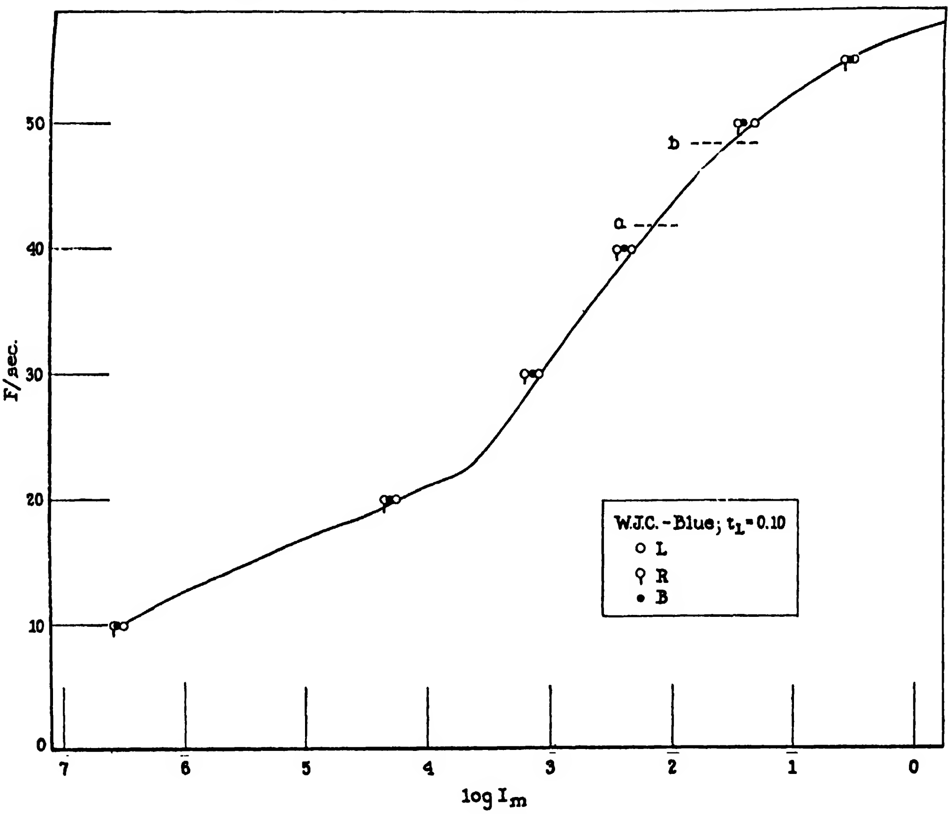

FIG. 13. Data for the comparison of *L*, *R*, and *B* flicker recognition thresholds (W. J. C.) with blue light, light-time fraction $t_L = 0.10$. The "foveal" type of end-point appears at the level *a*; the blue color threshold is at *b*. The curve (*L*) is that obtained from other, more complete series under these conditions (*cf.* Crozier and Wolf, 1940–41 *d*).

binocular fusion the observer sees two illuminated squares, each centrally fixated. The differential *R*, *L*, brightness is then obvious, but *both* fields are seen to flicker simultaneously *at the critical flash intensity characteristic of the binocularly fused image* (for the same *F*), whether this be below or between the *R* and *L* values. Central integration of effects simultaneously arising from the two eyes must consequently be taken as proved, and as independent of subjective fusion of the images.

The possibility existed that *R*, *L* differences might perhaps be magnified

under certain conditions. Since the whole F-log I contour is enlarged and shifted toward lower flash intensities when the light-time fraction is reduced (Crozier and Wolf, 1940–41 *c*, *d*), and also by using blue light, an experiment was done with blue light (Wratten Filter No. 47) and a flash cycle with 0.10 light-time. The absolute scatter of the determinations of critical flash intensity is also lower under these conditions. The data are given in Table II and in Fig. 13. W. J. C. was used since in the data with white light (Fig. 5) his B figures were more nearly intermediate between the R and L. It is shown in Fig. 13 that the separation of R and L

TABLE II

Size and location of retinal image as in Table I—6.13° square, centrally fixated; blue light (Wratten Filter No. 40); light-time = 0.10 of the cycle time. Left eye (L), right eye (R), and binocular (B) determinations (n = 10) of mean critical flash intensity for flicker and of P.E.$_{1I_1}$; observer, W. J. C. The intensities (*ml.*) are in terms of a photometric match against white light (below the color threshold). See text and Fig. 13.

F	L		R		B	
	log I_m	log P.E.$_{1I_1}$	log I_m	log P.E.$_{1I_1}$	log I_m	log P.E.$_{1I_1}$
per sec.						
10	$\bar{7}.5226$	$\bar{9}.8619$	$\bar{7}.4479$	$\bar{9}.8740$	$\bar{7}.4709$	$\bar{9}.8716$
20	$\bar{5}.7765$	$\bar{6}.2474$	$\bar{5}.6696$	$\bar{7}.9977$	$\bar{5}.7105$	$\bar{7}.9564$
30	$\bar{4}.9384$	$\bar{5}.3463$	$\bar{4}.8157$	$\bar{5}.0918$	$\bar{4}.8822$	$\bar{5}.2144$
40	$\bar{3}.6863$	$\bar{4}.0867$	$\bar{3}.5652$	$\bar{5}.8545$	$\bar{3}.6207$	$\bar{5}.8631$
50	$\bar{2}.6966$	$\bar{3}.0703$	$\bar{2}.5533$	$\bar{4}.7211$	$\bar{2}.6050$	$\bar{4}.8846$
55	$\bar{1}.5232$	$\bar{2}.0339$	$\bar{1}.4436$	$\bar{3}.8366$	$\bar{1}.4882$	$\bar{3}.6612$

is of exactly the same general sort as with white light (Fig. 5), and that B is again intermediate. It is important that under the conditions of this particular test the blue color threshold is not reached until just below F = 50 on the curve, and the typical "foveal" appearance of the flicker recognition point not until *ca.* F = 43 along the curve. A fuller analysis of these indications is attempted in following papers, but their significance for the classical use of ordinary criteria for rod and cone function is highly interesting. For present purposes the measurements show that the relationships between L, R, and B measurements already discussed for white light and $t_L = t_D$ are in fact independent of wave-length composition of light and of the light-time fraction, and of the *brightness* level.

Analysis of the variation of I_c, the critical flash intensity, and of the scatter of the indices of this variation, supplies further important criteria which the theory of neural integration for binocular flicker must satisfy.

VI

The recognition of flicker is a form of intensive discrimination; the fact that at high flash frequencies the critical flash frequency does not correspond, subjectively, to the physically impressed frequency is no bar to this interpretation. One of the aspects of the homology of the flash intensity with ΔI_0 and ΔI as ordinarily determined is the parallel way in which I_e, like ΔI_0 or ΔI, is directly proportional to its own index of variation (σ_I or P.E.$_I$), as shown for many series of measurements (Crozier, 1935–36; Crozier and Holway, 1937, 1938, 1939–40; Crozier, Wolf, and Zerrahn-Wolf, 1937–38 *a*). There are two aspects of the interdependence of I_e and P.E.$_I$, namely the mean magnitude of the proportionality constant and the manner of distribution of the values found in the band of slope = 1 relating log P.E.$_I$ to log I_m. For sufficiently homogeneous data the distribution is such that the line giving the mean value of the proportionality constant divides the log P.E.$_I$ width of the band *arithmetically* in half. For non-homogeneous data with a single observer, such as result from the massing of observations taken over a period of some days, this line simply divides the log P.E.$_I$ span equally. The position of the median line and the positions of its margins can be objectively determined by projecting the positions of the points along a 45° slope to a common ordinate and determining the mean and the S.D. for the frequency distribution of the intercepts (*cf.* Crozier and Holway, 1937, 1938). For the two observers the mean P.E./I_m here obtained is (W. J. C.) a little lower and (E. W.) a little higher than in the older series with other apparatus (Crozier, Wolf, and Zerrahn-Wolf, 1937–38 *b*). (The absence of a "break" in the present variation plot is correlated with the small rod group.)

The study of the properties of the variation of ΔI and of ΔI_0 (Crozier and Holway, 1938; 1939–40) has shown that this variation, under uniform conditions of test, has properties which must be regarded as an organic product of the performing system under test. These considerations reappear in the data of the present experiments. From measurements of ΔI at different levels of I_1 it was shown (Crozier and Holway, 1939–40) that with monocular measurements, at different areal exposures and for different wave-lengths, $\sigma_{1\,\Delta I}$ and ΔI_m were in the same statistically constant proportion and slightly *lower* than for corresponding measurements made binocularly. For the binocular determinations, however, ΔI measurements *at a given level of* I_1 are lower than the mean of the values for the two eyes individually (Crozier and Holway, 1939–40), in a mean ratio a little less than the 1.41 obtained for "absolute" thresholds with the same apparatus and general procedure (Crozier and Holway, 1938–39 *b*).

The variation data in Table I are plotted in Figs. 14 and 15. It is shown, in the first place, that the proportionality constants for monocular P.E.$_{.I}$ *vs.* I_m are not the same for the two eyes, being a little lower for R; the breadth of the scatter band is a little greater, however, for R. In each case the mean ratio for the binocular measurements B is definitely lower than that for either R or L taken alone.

The mean values of P.E.$_{.1}/I_m$ are for W. J. C.: R, 3.62; L, 4.67; of σ_1 of the sets, R, 1.45, L, 1.41; the B mean ratio is 2.99, its $\sigma_1 = 1.34$. The average of the ratios for R and $L = 4.15$, which is 1.39 times the value for B. (These values are all lower than found for determinations of ΔI and ΔI_0 under the same conditions with this observer.)

With E. W. the corresponding values for the means are, for P.E.$_{.1}/I_m$: R, 9.31, L, 10.06, B, 6.68; the average for R and L is 1.45 times greater than the value for B, as compared with 1.39 for W. J. C. For R and L, σ_1 is 1.466 and 1.471, for B, 1.34.

The values of $\sigma_{1\log \text{P.E.}_1}$ for W. J. C. and E. W., which are the proper basis of comparison of the monocular and binocular dispersions since the data are non-homogeneous to the extent that they comprise compound fluctuations, are in the $(L + R)/2\ B$ ratio of 1.25 and 1.37.

Thus both the mean value of the precision and of the scatter of the determination of I_m, with the intensity level automatically corrected out, is definitely less for the binocular measurements than for either of the contributory uniocular processes taken singly. The value for the B data is found to stand in the ratio of 1.39 (W. J. C.) and 1.45 (E. W.) to that for the average values for the respective right and left eyes. It is very doubtful if either of these values departs significantly from their mean, 1.42. The variation data in Table II, for W. J. C. with blue light and $t_L = 0.10$, tell essentially the same story, although they are not sufficientiy numerous for analysis. This is the kind of result clearly to be predicted on the basis that discriminatory precision is a consequence of the number of elementary units involved in a *statistical* discrimination (Crozier, 1936), and if in the binocular measurements the numerical potency of these *discriminatory* elements is doubled, the precision is accordingly increased in the ratio $\sqrt{2}$ to 1.

We have to note that only when the procedural errors are held reasonably constant, and when the correlation between I_m and σ_{1I} can be used to exhibit their relationship independent of intensity, can this type of demonstration really be made. It is important to remark, however, in view of the fact that different operators were in control of the instrument for the E. W. and W. J. C. series, that the quantitative relationship between

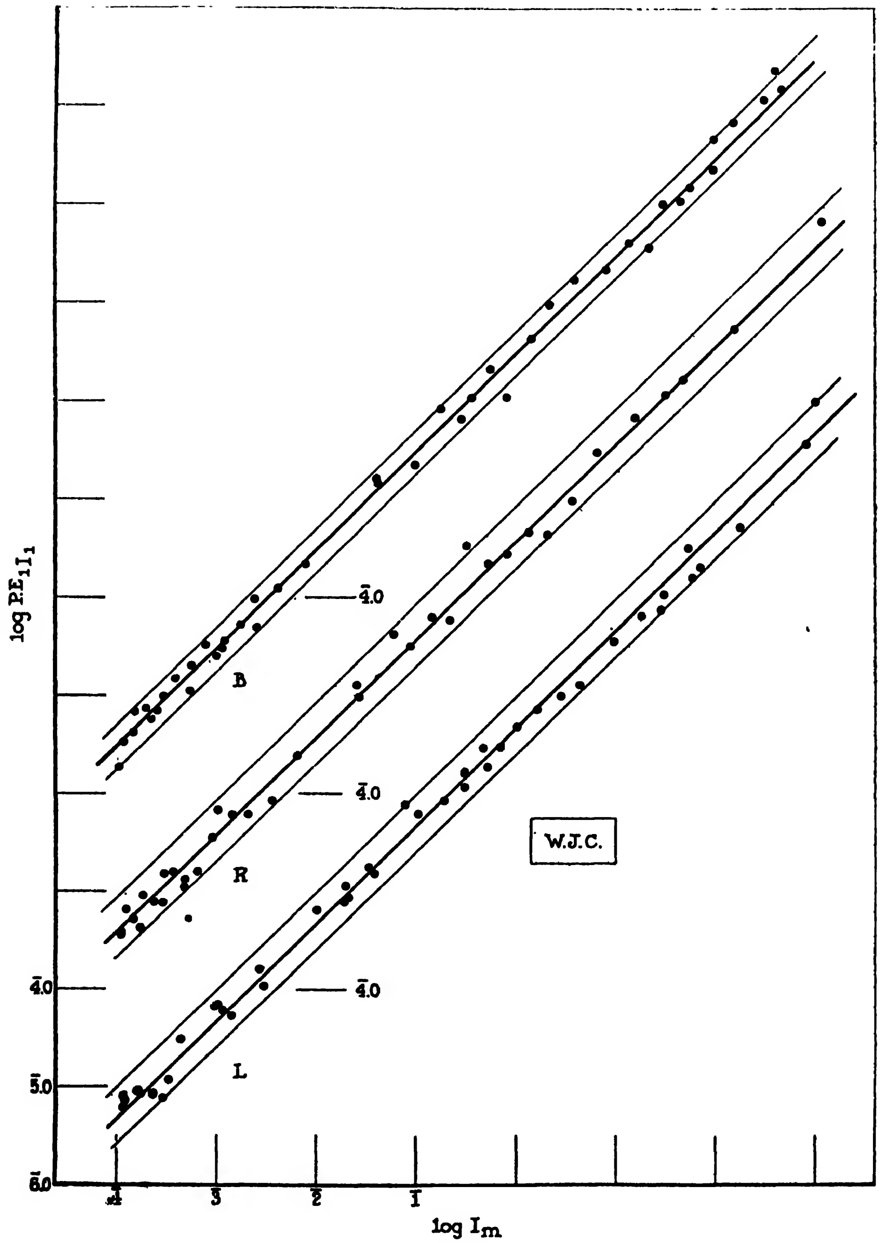

FIG. 14. The dispersion indices for the values of I_1 averaged to give I_m are rectilinearly related to I_m; log P.E.$_{\cdot 1}$ $_{I_1}$ *vs.* log I_m gives a band of statistically constant height and slope = 1. Data for W. J. C. (Table I); the bands are separated vertically for clearness. The *proportionality constant* is *less* for *B* (in the ratio 1.39 to 1), and the relative scatter of P.E.$_{\cdot 1}$ $_{I_1}$ is less. See text.

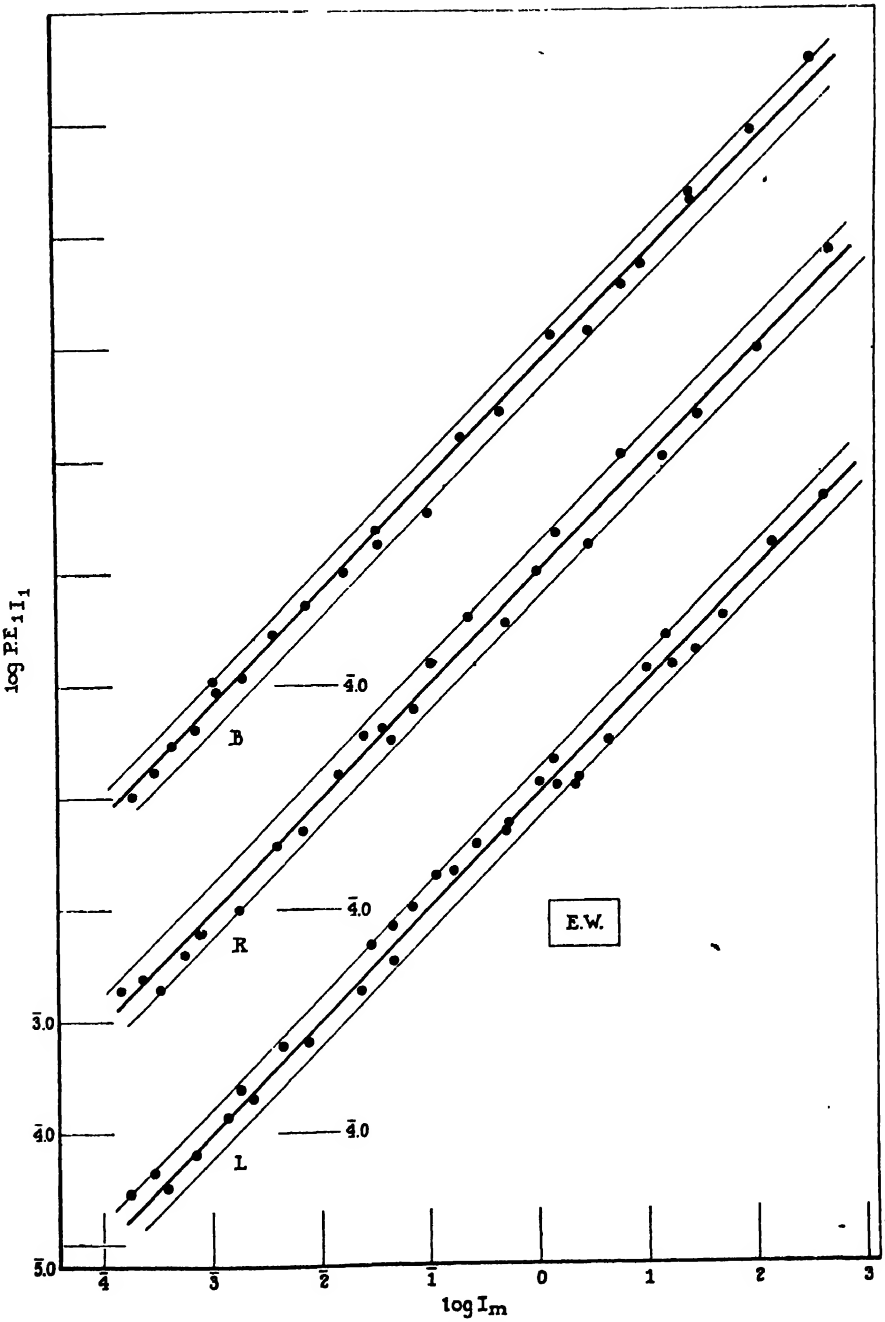

FIG. 15. Variation data for E. W. (Table I); as in Fig. 14; see text.

$P.E._{1I_1}$ and I_m is not (within reasonable limits, at any rate) a function of the manipulator. Nor, as we know from adequate tests, is it modified essentially by changes in the procedure used in approaching the end-point. A chief possible source of such modification lies in the level of adaptation adopted as the standard from which to approach the end-point, and in the rate of this approach. For W. J. C. the standard method was to increase the flash intensity from *ca.* 0.18 log unit below the critical region. The mean value of I_m can be made 0.10 log unit lower by beginning from a lower level of adaptation, at 0.90 log unit below the critical region. This is, of course, to be expected, but the important fact is that $P.E._{1I_1}$ then still has the same relationship to I_m. With E. W. each measurement was begun from a level of intensity proportionately lower than with W. J. C.; but this cannot explain the differences found in their variability functions.

VII

The evidence described may now be considered in reference to the theory of binocular summation in symmetrical flicker. The data show that there are systematic differences between the binocular flicker recognition contours and the contributory uniocular contours determined separately. The differences cannot be adequately described simply by saying that the *B* data are intermediate between those for *R* and *L*; the shapes of the contours differ. For the cone segments the parameter τ', the log I_m abscissa of inflection of the curve, is rather precisely intermediate between those for *R* and *L*. The analyzed properties of the rod segments show their forms to result from complex interaction between cone and rod elements of effect, in the sense that progressive increase of cone effects inhibits the action of rod effects. The rising branch of the pure rod contribution to the duplex curve, obtained by deducting the cone effect in the region of their overlapping, is found to show the *B* curve following pretty closely that for the eye (*R*) with lower I_0. That a real neural integration is involved in the production of these findings is shown by the synchronous behavior of the end-point for non-binocularly fused images in the two eyes.

We have also the fact that the binocular brightness is certainly greater at all critical fusion levels than that for either eye alone. The *R* images at fusion were subjectively brighter than the *L* and I_0 was lower. The relations between the *R*, *L*, and *B* critical intensities are the same when the determinations are made with blue light in a flash cycle with 10 per cent light-time. Under these conditions the level of brightness for the *F*-log I_m curve as a whole is very low, the fusion color threshold being at *ca.* $F = 50$, but I_0 is far below that for the white light cases already con-

sidered. Consequently, the critical flash intensity cannot be considered to be determined by the general or relative brightness.

The further and quite significant general fact provided by the measurements is revealed in the relationships of the mean values of I_e to their indices of precision,—or, more exactly, the rectilinear relations of P.E.$_{1I}$ to I_m. For B, the relative scatter of I_1 is less than that for R or L, in the general ratio of 1:1.43. Moreover, the scatter of the values of P.E.$_1$ is less. Now we know (Crozier and Wolf, 1940–41*d*) that when the light-time ratio is varied the scatter of P.E.$_1$ as a function of I_m is less the larger the light-time fraction; the relationship is rectilinear. In this case the P.E.$_{1I_1}$ span is directly proportional to the value of $F_{\text{max.}}$, a fact confirmed by tests in different parts of the retina as well (Crozier and Wolf, 1940–41*d*). This sort of thing cannot be entirely a matter of statistical dependence or accident, since in general theory σ and σ_σ must be in simple proportion. When σ_σ, corrected for I, is found to be directly proportional to $F_{\text{max.}}$, as in the experiments involving retinal position and the light-time fraction, with *area* of image constant, the notion arises that the breadth of the variation band is decreased with increase of the brightness level and with decrease of the total population of elements ($d\,F/d \log I$) involved. There is some support for this in corresponding data with colored lights, which we describe in a subsequent paper. But it is clear that the factor of subjective brightness level and the factor of "size of population" do not necessarily work concurrently. This is abundantly shown by the colored light data. For the B, R, L cases the B fusion brightness is greater, but $F_{\text{max.}}$ is only slightly or not at all increased; in conformity to the increase of brightness, σ_{I_e} decreases, and σ_I/I_m is reduced as $1:\sqrt{2}$—although when monocular brightness is increased by increasing t_L it does not change. At the same time, the B subjective fusion brightness is certainly not doubled, and we suspect that its ratio to the mean of R and L fusion brightnesses is a function of the intensity level.

To rationalize these somewhat confusing relationships it is necessary to suppose that *brightness* is one kind of sensory effect, while $F_{\text{max.}}$ measures another. The relations between them are complex. The values of P.E.$_1$ and the scatter of P.E.$_1$ are not determined by the brightness level for the function as a whole, and are not determined by $F_{\text{max.}}$, although in different circumstances they may appear to be correlated. The fact that the two statistical indices are independent of I_m along any one contour can be best understood on the basis that in the determination of the critical intensity, at any level of F, the whole population of elements potentially available under the conditions is actually at work. This is the essence of the con-

ception of statistical fluctuation used in the derivation of the expectation that the form of the contour will be given by a probability summation (*cf.* Crozier 1937; 1940 *a*, *b*). For binocular flicker the number of these elements is doubled in some fashion, as the variation indices prove, and the fusion brightness is somewhat increased (correlated with a decrease in the *scatter* of $P.E._{1\,I}$), but this does not materially increase $F_{max.}$. In other words, the potential effectiveness of each element is doubled, but the total number is pretty much the same. This is not dependent on subjective fusion of the images from the two eyes.

The fact that the probability summation effectively describes the flicker data and their modifications under different conditions of retinal area, location, wave-length of light, light-time fraction, and temperature, in a wide diversity of animals, and for monocular and binocular presentation, is of course a potent argument for the propriety of using it. In the various experiments made with arthropods, lower vertebrates, and birds, surrounded by a rotated cylinder with vertical stripes, binocular stimulation is necessarily used. (This of course does not mean binocular fusion of the field of regard.) Certain particular problems arising in this connection, by reason of the fact that the animal is free to move about within the cylinder, have been discussed on the basis of experiments with the crayfish *Cambarus* (*cf.* Wolf, 1940; Wolf and Crozier, 1940–41). If the essential dynamical properties of such data are determined centrally, and they must be so regarded in the binocular instance, then two possibilities exist: either the properties of the uniocular data are also determined centrally, or else the nature of the measurements has a similar character whether determined centrally or peripherally. The demonstration of a statistical basis for the nature of the data is of course by itself consistent with either possibility. The composition of two probability distributions gives another probability distribution; the Gaussian function is apparently the *only* one having this property (Cramér, 1937). Unquestionably this is the source of its general capacity to account for the data in these complex situations.

There arises naturally at this point the question as to the manner in which the central nervous composition of two independently produced uniocular effects could be expected to show itself. In the interpretation of complex visual effects considerable general use has been made of the terms "inhibition" and "summation." It is preferable to speak rather of *integration*, since this may be done without theoretical prejudice. This conception avoids the troubles arising in the situation depicted in Figs. 4 and 5; inhibition with respect to one eye is often summation with respect to the other; the intermediate value of the B cone τ' (and the B $\sigma'_{\log I}$) certainly

denotes *integration* rather than anything else. Difficulties are also avoided when dealing with the rod-cone overlap: if our analysis of the situation is sound, inhibition of some rod effects is simultaneously accompanied by summation of cone effects with the remainder.

The determination of visual threshold intensities shows that for the "absolute" threshold the effect of *doubling area* in one retina is qualitatively like that produced by viewing the *same area* simultaneously with both eyes under conditions of binocular fusion (Crozier and Holway, 1938–39 *b*). For understandable reasons, in part (*i.e.*, fixation), the binocular mean *variation* is increased, but the ratio of mean $(\Delta I_0)_B$ to the mean for L and R is not certainly different from 1.41. There is reason to believe that this ratio may be a function of the exposure time; when ΔI is obtained for finite levels of I_1 it is influenced only slightly by the intensity level; the effect on ΔI of enlarging the retinal area illuminated is quantitatively the same for binocular and uniocular presentation (Crozier and Holway, 1939–40: Crozier, 1940 *a*). It is greater than the ratio obtained for symmetrical doubling of area on one retina. The examination of the exposure time function shows, on the other hand, that within the fovea enlargement of area of test-patch beyond a certain limit brings about an increase of variability in the population of cone effects (Crozier, 1940 *a*). The same result appears in F-log I contours when retinal location or test-patch area are changed to include different sized populations of cone effects (Crozier and Wolf, 1940–41 *d*): $\sigma'_{\log I}$ for the cone curve is increased when the cone population is *reduced*. For the rod populations the *opposite* result is obtained: *enlargement* of the rod population of units brings with it an increase in $\sigma'_{\log I}$ for the rod curves (Crozier, 1940 *c*), as already shown for dark adaptation (Crozier, 1940 *b*) under different conditions modifying the size of the dark-adapting population of elements. The homologous result in the two sets of flicker experiments with different areas that we have earlier discussed (section IV) is confirmatory. Both the enlargement and the reduction of $\sigma'_{\log I}$ as a result of increasing the number of the respective *retinal* units, by different methods of modification of the conditions of test, signify interaction and integration of neural effects at some level, but cannot reasonably be discussed in terms of inhibition and summation.

The closest analogy for the basic binocular effect in flicker is found in the data on binocular *vs.* monocular ΔI_0 and ΔI. The ratio is about 1.41, but the quantitative result of enlarging retinal area illuminated is the same in both cases (Crozier and Holway, 1938–39 *b*, 1939–40). This proves that in the discrimination of I_2 from I_1 the binocular effect is doubled, although for a given ΔI the size of $\sigma_{1\Delta I}$ is not much affected, if at all. It has

been possible to show (Crozier, 1940 *b*) that ΔI is really determined by the size of the population of effects available for further excitation, under the conditions given, so that $1/\Delta I$, the measure of excitability at any level of I_1, is a declining probability integral in terms of log I_1. In the flicker case the magnitude of the level of "sensory effect" must be taken as directly proportional to F; but this measures the discrimination of the effect of flash intensity from the effect of flash after image (*cf.* Crozier, Wolf, and Zerrahn-Wolf, 1936–37 *b*). If as a consequence of binocular regard the flash effect is increased, so also is its after influence. Consequently one must expect, it seems, on this basis, that the log critical flash intensity (τ') for activation of one-half the total number of elements should appear as the *mean* of those for the two uniocular components,—and, in the case of the raw rod curves, the B curve for the observed result should be the average of the two composite rod effects, which is seen. On the other hand, the *precision* with which the light-dark discrimination is statistically made should be, for a given value of I_o, increased by the factor $\sqrt{2}$ if the potency of each element concerned in making it is doubled. This the data show to be the fact.

VIII

SUMMARY

Comparison of monocular and binocular critical flash intensities for recognition of flicker, using a centrally fixated square image subtending *ca.* 6.13° on a side (white light), shows that for the cone segment of the response contour the inflection point of the probability integral correlating flash frequency F (for symmetrical flicker) and log mean critical flash intensity I_m is with the binocular measurements exactly intermediate between those for each eye separately. This does not mean that in general the data are intermediate; they are not; the binocular asymptotic $F_{max.}$ agrees with or lies above the greater one of the two uniocular curves. The entire contour must be considered for valid intercomparisons, as is also true if homologous curves for different observers are to be compared. For the measurements in the predominantly rod region the binocular data are more or less intermediate. The rod curves result, however, from the integrative interplay of rod and cone effects for which the intrinsic curves overlap. The resultant rod curve as measured is determined by the partial inhibition of rod effects by cone effects, and by the summation of the remaining rod contributions with those labelled cone in origin. It is pointed out that in this respect, as in others, it is desirable to consider the rôles of retinal area,

and location, from the standpoint of *integration* of neural effects. These phenomena are essentially independent of the light-time fraction and of the spectral (λ) quality of the light used.

For binocular, as for uniocular excitation, the normal probability summation provides an efficient general description, under diverse conditions of size and location of retinal image, wave-length composition of light, light-time cycle-fraction, and kind of animal. It is pointed out that this is the only function abstractly likely to exhibit this kind of efficiency.

That a summation of veritable effects independently generated by simultaneous, symmetrical uniocular excitation does occur in the recognition of flicker is specifically demonstrated by the fact that for a given mean critical flash intensity the associated variation is lower for binocular than for either or the average of the single-eyed presentations,—and in the ratio not statistically different from 1:1.41; the relative scatter of the binocular indices of dispersion is also reduced below the uniocular. Since the mean variation of the critical intensity is statistically in a constant ratio to I_m, in appropriately homogeneous series, independent for example of the brightness level and of the light-time fraction, this signifies an essential doubling of the effectiveness (potential) of each of the elements concerned in the discrimination of flicker when binocular excitation is concerned, although the total number of these elements is only slightly or not at all affected. The potential in question is not exclusively correlated with subjective brightness-at-fusion, which is, however, increased with binocular regard.

CITATIONS

Cramér, H., 1937, Random variables and probability distributions, Cambridge Tracts Mathematics and Mathematical Physics, No. **36**, London, Cambridge Press.

Crozier, W. J., 1935, Déterminisme et Variabilité, Paris, Hermann et Cie. 1935–36, *J. Gen. Physiol.*, **19**, 503. 1936, *Proc. Nat. Acad. Sc.*, **22**, 412; 1937, **23**, 71; 1940 *a*, **26**, 54; 1940 *b*, **26**, 334; 1940 *c*, in press.

Crozier, W. J., and Holway, A. H., 1937, *Proc. Nat. Acad. Sc.*, **23**, 23; 1938, **24**, 130; 1938–39 *a*, *J. Gen. Physiol.*, **22**, 341; 1938–39 *b*, **22**, 351; 1939–40, **23**, 101.

Crozier, W. J., and Pincus, G., 1929–30, *J. Gen. Physiol.*, **13**, 57.

Crozier, W. J., and Wolf, E. W., 1938–39, *J. Gen. Physiol.*, **22**, 463. 1939, *Biol. Bull.*, **77**, 126. 1939–40 *a*, *J. Gen. Physiol.*, **23**, 1; 1939–40 *b*, **23**, 143; 1939–40 *c*, **23**, 229; 1939–40 *d*, **23**, 531; 1939–40 *e*, **23**, 667; 1939–40 *f*, **23**, 677; 1940–41 *a*, **24**, 317; 1940–41 *b*, **24**, in press; 1940–41 *c*, **24**, in press; 1940–41 *d*, **24**, in press; 1940–41 *e*, **24**, in press.

Crozier, W. J., Wolf, E., and Zerrahn-Wolf, G., 1936–37 *a*, *J. Gen. Physiol.*, **20**, 363; 1936–37 *b*, **20**, 393; 1937–38 *a*, **21**, 17; 1937–38 *b*, **21**, 203; 1937–38 *c*, **21**, 223; 1937–38 *d*, **21**, 313; 1938–39 *a*, **22**, 311; 1938–39 *b*, **22**, 451; 1938–39 *c*, **22**, 555.

Hecht, S., 1937, *Physiol. Rev.*, **17**, 239.

Sherrington, C. S., 1902, *Proc. Roy. Soc., London*, **71**, 71. 1904, *Brit. J. Psychol.*, **1**, 26. 1906, The integrative action of the nervous system, New Haven, Yale University Press.

Wolf, E., 1940, *Anat. Rec.*, **78**, 92.

Wolf, E., and Crozier, W. J., 1940–41, *J. Gen. Physiol.*, **24**, in press.

TRANSVERSE IMPEDANCE OF THE SQUID GIANT AXON DURING CURRENT FLOW

By KENNETH S. COLE and RICHARD F. BAKER

(From the Department of Physiology, College of Physicians and Surgeons, Columbia University, New York, and The Marine Biological Laboratory, Woods Hole, Massachusetts)

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INTRODUCTION

During the passage of an impulse in Young's squid giant axon preparation (Young, 1936), a considerable increase in the membrane conductance was found but relatively little if any change in the membrane capacity (Cole and Curtis, 1939). The conductance increase was interpreted as a measure of the increase of ion permeability which is commonly assumed to be a part of the nerve impulse.

It is generally believed that when a current flows through a nerve membrane, the ion permeability is increased at the cathode and decreased at the anode. If this is true and the interpretation of the impedance change obtained during activity is correct, there should be an increase in the membrane conductance at the cathode and a decrease at the anode without a change in the membrane capacity during current flow. With these assumptions, an apparent contradiction is then found in the observations during the propagation of an impulse. During the foot of the action potential (before the point of inflection in the rising phase) the simple cable theory requires that the direction of positive current flow be outward across the membrane, or cathodic. This would require an increase of membrane conductance, which was not found. At the point of inflection, where the current flow reverses from outward to inward, there should be a decrease of the membrane conductance to a value less than that at rest. But it was at about this point that the large and rapid increase of the membrane conductance was observed. The first step in resolving these contradictions is to investigate the validity of the assumptions. For this it is necessary to determine the dependence of the membrane conductance upon the direction and magnitude of the membrane current flow in the resting axon.

The polarizing current could be applied suddenly for sub-rheobase cathode

and all anode polarizations, but the results of this procedure might be misleading when excitation and propagation take place at the cathode. Although the nature of the "depolarization" which occurs at the onset of activity is not known, it is often thought of as a breakdown or "relaxation" process. It would not be at all unreasonable, from analogy with nonliving systems, to expect that a polarizing current might be able to maintain a depolarization originally set up by excitation. Consequently, to avoid ambiguity, excitation should be avoided by increasing the polarizing current to its maximum value slowly. Then the terminal impedance change could be compared with that obtained after an excitation took place at the sudden make of the current to determine whether or not a depolarization could be maintained and whether or not the precaution of a slow rise was necessary. This question can also be answered, by gradually polarizing the nerve without excitation, and then allowing an impulse initiated at a distance to pass through the polarized region.

After the investigation of these steady effects of polarization, before and after activity, the next step is to trace the time course of the sub-threshold changes at the start of polarization. If we assume that before excitation the membrane has reproducible and reversible electrical characteristics, it should be possible to determine them and so explain the sub-threshold observations. The impedance changes at the site of excitation and during the passage of an impulse through a polarized region are of obvious importance in the quantitative description of the processes of excitation and propagation in nerve. It may be expected, however, that an explanation of these impedance data will itself be a rather complete theory of nerve activity.

Material and Apparatus

A description of the material and apparatus was given in the previous paper (Cole and Curtis, 1939) and only the modifications and extensions required in the present experiments will be given in detail.

Axon, Measuring Cell, and Bridge.—The giant axon in the hindmost stellar nerve of the squid, *Loligo pealii*, was dissected out and placed in the measuring cell. This cell was a strip of insulating material with a groove, *AA* (Fig. 1) 550 μ square, just large enough to accommodate the axon, cut in the top and covered with a glass cover slip. Sea water was circulated past the axon and the whole was usually kept at a temperature of about 4°C. Two platinized platinum impedance electrodes 550 μ square were mounted flush with the sides of the groove and facing each other. The transverse alternating current impedance of the axon was measured between these electrodes over a frequency range from 1 kc. to 500 kc. in a Wheatstone bridge (Cole and Curtis, 1937) with heterodyne, amplifier, and cathode ray oscillograph for detector. The measuring current through the cell was kept as low as possible without undue sacrifice of overall sensitivity, and in all cases the bridge balance was independent of this current.

Polarizing Circuit.—The current, or polarizing, electrodes were spaced as far apart as possible along the length of the axon, to separate the anode and cathode regions of membrane current flow and allow individual investigation of each region. The maximum membrane current density is to be found directly under an electrode and since it is approximately uniform only under a short electrode, the impedance should be measured through one of the polarizing electrodes. The other impedance electrode could then be placed in the extrapolar region for a longitudinal measurement but this involves a second membrane or an inactive end, and, in addition, a rather clumsy theoretical analysis. It is simpler to use a transverse impedance measurement where one-half of the polarizing current enters at each impedance electrode, *E*, through a resistance, *R*, and the total current leaves the measuring cell at the third, distant, electrode as indicated in Fig. 1. The two resistances, *R*, were 10,000 ohms each and this value

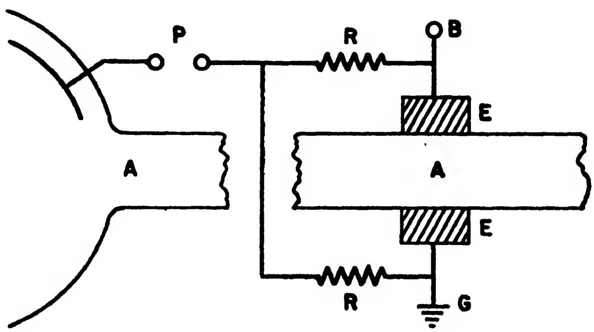

FIG. 1. Circuit for impedance measurements during current flow. Axon was placed in trough *A A* and transverse impedance measurements made between electrodes *E E* by the alternating current bridge connected at *B* and ground, *G*. The polarizing current from source *P* divided and flowed into this trough and axon through the two resistances *R R* and the electrodes *E E*. The current returned through the distant electrode at the left hand end of the trough.

was sufficiently large practically to eliminate differences of current flow in the two paths caused by asymmetries of the impedance electrodes, axon position, and membrane potential. Since a part of the bridge current flows through these two resistances in series, the sensitivity of the measurement of the axon impedance was decreased but this loss was not excessive. An approximately uniform current density under the electrodes was expected because the length of the electrodes, 0.55 mm., was considerably less than the "characteristic length" of about 3 mm. found for this axon (Cole and Hodgkin, 1939). It was found experimentally that a current flow from the impedance electrodes to two remote electrodes, one at each end of the cell, produced the same impedance change as was produced when this same current flowed to a single electrode at one end of the cell. Since this procedure was equivalent to a 50 per cent reduction of the effective electrode length, the polarizing current density in the membrane was essentially uniform in the region where the impedance measurements were made.

The magnitude of the polarizing current was made practically independent of the characteristics of the electrodes and of the nerve, by applying a sufficiently high potential

through a series resistance of 150,000 ohms to the terminals, P. The currents were varied up to a maximum of about 1 milliampere and were measured directly with a meter and also calculated from the resistances and potential.

The current was controlled by the opening of three contacts operated from motor driven cams, and the oscillograph sweep circuit was controlled by a fourth contact. The complete cycle of this contactor was usually repeated at intervals of about 1 second. Sudden on and off currents were easily obtained, and use of a variable shunt condenser gave "exponentially blunted"[1] currents with a range of time constants up to 100 msec. With well platinized impedance electrodes, the effect of the maximum polarizing current on the measured impedance of the cell filled with sea water was less than 0.1 per cent.

EXPERIMENTAL

The first experiments were made with an exponentially blunted polarizing current, followed by an equal and opposite current of the same duration and form, as shown in Fig. 2, to minimize the injury to the axon. For this

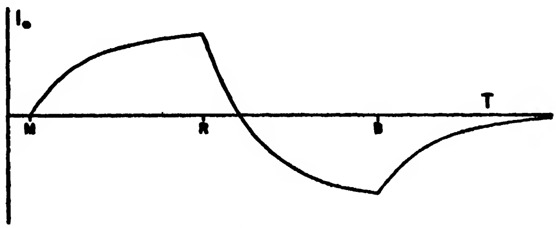

FIG. 2. Schematic drawing of the exponentially blunted current flow, I_0, applied in the sequence: make, M, reverse, R, break, B, *vs.* time, T.

the contactor applied the potential to the resistance and capacity polarizing circuit for about 200 msec. in the sequence on-reverse-off. The Wheatstone bridge was first balanced at each frequency without the polarizing current and then a change of the axon impedance caused by current flow gave an output voltage from the bridge and a band on the oscillograph. Typical records are shown in Fig. 3 for 20 kc. and 125μ amp. maximum current. Soon after the cathode make, *m* in Fig. 3 *a*, there was a short impedance decrease associated with the excitation. Then a slow decrease of the impedance followed, in both *a* and *b* of Fig. 3, as the current increased towards its maximum value. After *r*, where the reversal was started, the impedance first increased to return to the resting value and then increased still farther to again unbalance the bridge, but in the opposite direction, during the anode current flow. After *b* the anode current decreased towards zero and the impedance decreased correspondingly to leave the

[1] This term is used in preference to "exponentially rising" to describe the time course given by $1 - e^{-t/T}$ and shown in the initial rise of Fig. 2.

bridge balanced finally. The bridge unbalances were found in the reverse order when the anode was applied first, as is seen in Fig. 3 *c*, and a double excitation took place at the reversal. The final magnitudes of the impedance changes at the cathode and anode are the same, whether the cathode (Fig. 3 *a* and *b*) or anode (Fig. 3 *c*) was applied first. Within the limit of sensitivity, for all frequencies and currents, the change of impedance at the cathode was a decrease from the resting value and that at the anode, an increase. For this axon, 125 μamp. with a time constant of 50 msec. gave excitation as shown in Fig. 3 *a*, and with an increase of the time constant to 80 msec. the same current was subthreshold (Fig. 3 *b*). It is apparent that the final impedance change was not appreciably altered by activity. As might then be expected, it was found that near rheobase, with suddenly applied polarizing current, the steady value of impedance change was independent of excitation, as seen in Fig. 4. In general, whether the current reached its maximum value very abruptly, or very slowly, the final change of impedance was the same. Also when a distantly initiated impulse was sent through the polarized region after the impedance change during current flow had become constant, the impedance would decrease during the passage of the impulse and then return to the previous level. We thus have evidence that the steady state effect of the polarizing currents employed is not altered by excitation. Consequently the use of exponential blunting to avoid excitation was unnecessary and sudden makes and breaks were subsequently used.

After the direct-reverse polarizations described, the impedance required several seconds to return completely to the resting value, irrespective of whether cathode or anode was applied first. This effect was entirely a result of the anode polarization and was not reduced by an equal cathode polarization either before or after. Since reversal technique apparently did not materially improve the survival of the axons, it too was an unnecessary complication and was abandoned in favor of the simple on and off polarizations, as shown in Fig. 4.

Having found a change in the transverse impedance during polarization, the next step was to determine which component of the axon was responsible for it. The procedure was the same as that used for the analysis of the impedance change during activity (Cole and Curtis, 1939) with the advantage that we are here dealing with something more closely approximating a steady state. At each frequency, the bridge was balanced to give the resting parallel resistance, R_p, and capacity, C_p(Cole and Cole, 1936; Cole and Curtis, 1937). The polarizing current was then applied for intervals long enough to allow the impedance change to reach a steady value

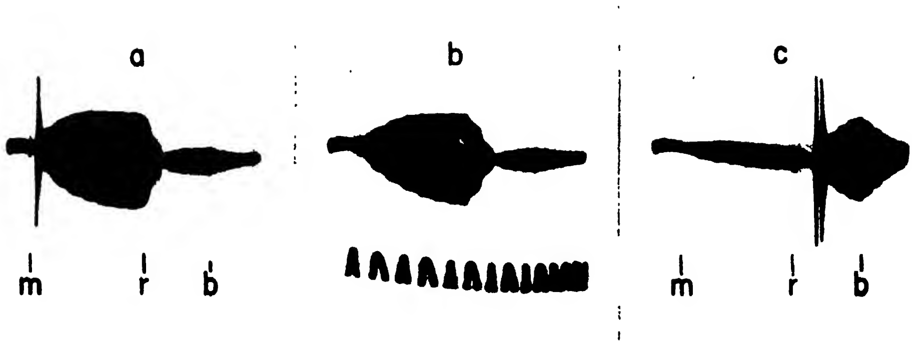

FIG. 3. Oscillograph records of bridge unbalance at 20 kc. caused by make, *m*, reverse, *r*, and break, *b*, sequence in exponentially blunted current flow of 125 μamp. (*a*) Cathode first, time constant 50 msec., showing excitation near beginning. (*b*) Cathode first, time constant 80 msec., sub-threshold. (*c*) Anode first, time constant 80 msec., showing double excitation at reversal. Exponential time scale indicated by 50 cycle timing wave below. Impedance changes at cathode, − 2 per cent; anode, +0.6 per cent, determined by calibration.

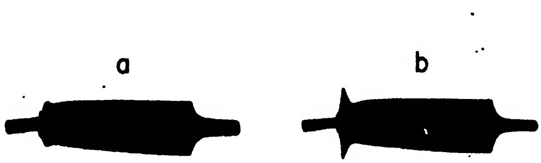

FIG. 4. Oscillograph records of bridge unbalance at 20 kc. caused by sudden make and break of cathode current flow. (*a*) Sub-threshold response for current just below rheobase. (*b*) Threshold response for current just above rheobase. Initial maximum is propagated.

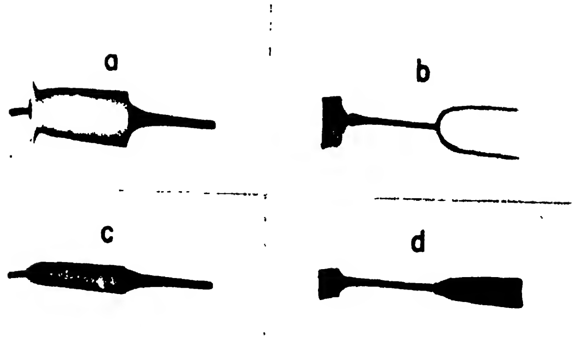

FIG. 5. Oscillograph records of bridge balance and unbalance at 20 kc. during current flow of 63 μamp. Cathode, (*a*) with bridge balanced at rest and (*b*) balanced during current flow. Impedance decrease, 2.0 per cent. Anode, (*c*) with bridge balanced at rest and (*d*) balanced during current flow. Impedance increase, 1.05 per cent.

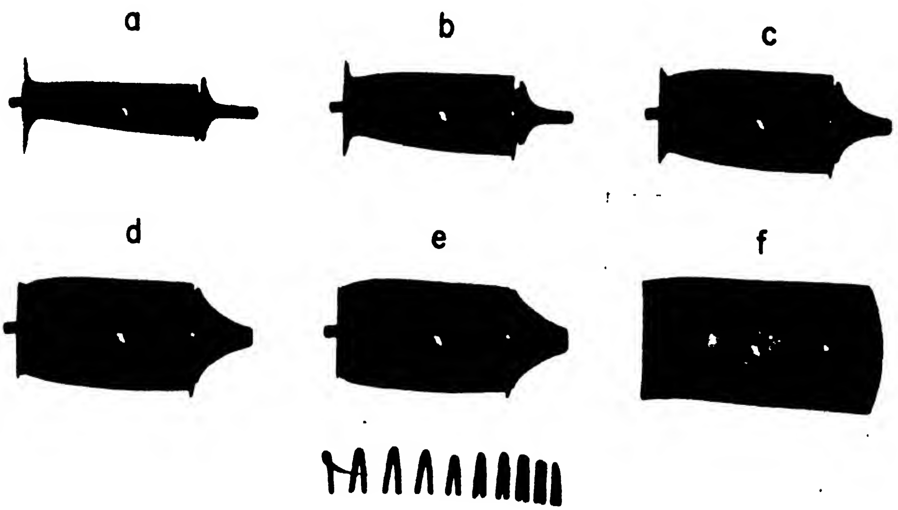

FIG. 6. Oscillograph records of bridge unbalance at 20 kc. caused by cathode current flow. Currents in μamp. are; (*a*) 105; (*b*) 210; (*c*) 315; (*d*) 420 and (*e*) 527. Transient impedance changes are caused by excitation under the impedance electrode, at the make, and by excitation propagated from the distant anode at the break. Calibration (*f*) is a 7.7 per cent impedance decrease. Maximum bridge unbalance during passage of a distantly initiated impulse without current flow (not shown) was −4.3 per cent. Timing wave, 100 cycles.

(Fig. 5 *a* and *c*). The bridge was then rebalanced for this steady value as shown in Fig. 5 *b* and *d*, to give new values of R_p and C_p.

The bridge balance usually could not be found in less than four or five applications of the polarizing current, and with large currents this process might cause irreversible changes. After sufficient bridge data had been taken to establish the nature of the impedance change, its dependence on the polarizing current was more satisfactorily obtained from oscillograph records of the bridge unbalance during a single polarization and a calibration as shown in Fig. 6.

Calculations and Results

The observed values of parallel capacity, C_p, are corrected for the polarization capacity of the electrodes and static capacity of the measuring cell (Cole and Cole, 1936; Cole and Curtis, 1937). The equivalent series resistance, R_s, and reactance, X_s, are then calculated from the parallel resistance, R_p, and capacity, C_p, by the equations

$$R_s = R_p/(1 + R_p^2 C_p^2 \omega^2), \qquad X_s = R_p^2 C_p \omega/(1 + R_p^2 C_p^2 \omega^2)$$

where $\omega = 2\pi$ times the frequency.

The values of R_s and X_s are then plotted as abscissae and ordinates to give the complex impedance locus (Cole, 1928, 1932). The loci shown in Fig. 7 are for an unpolarized and a cathodically polarized axon. It will be seen that the membrane phase angle and the infinite frequency resistance are unaltered by current flow. The impedance variation at a single frequency for a range of anode and cathode polarizing currents is shown in Fig. 8. These data indicate that the membrane capacity is practically unaltered and that the impedance change during polarization may be completely explained by a change of membrane conductance (Cole and Curtis, 1938).

The change of membrane conductance, ΔG, for a single value of polarizing current is computed from the extrapolated infinite frequency specific resistance, r_∞, and the extrapolated zero frequency specific resistances, r_0, for the unpolarized axon, and $\bar{r}_0$ for the polarized axon, by (equation 6, Cole and Curtis, 1938)

$$\Delta G = \frac{1}{a} \cdot \frac{\bar{r}_0 - r_\infty}{\bar{r}_0^2 - r_1^2} \cdot \frac{\bar{r}_0 - r_0}{r_0 - r_\infty}$$

where a is the radius of the axon, and r_1 is the specific resistance of the medium.

The data shown in Fig. 7 give $\Delta G = 0.03$ ohm^{-1} cm.$^{-2}$ for a cathode

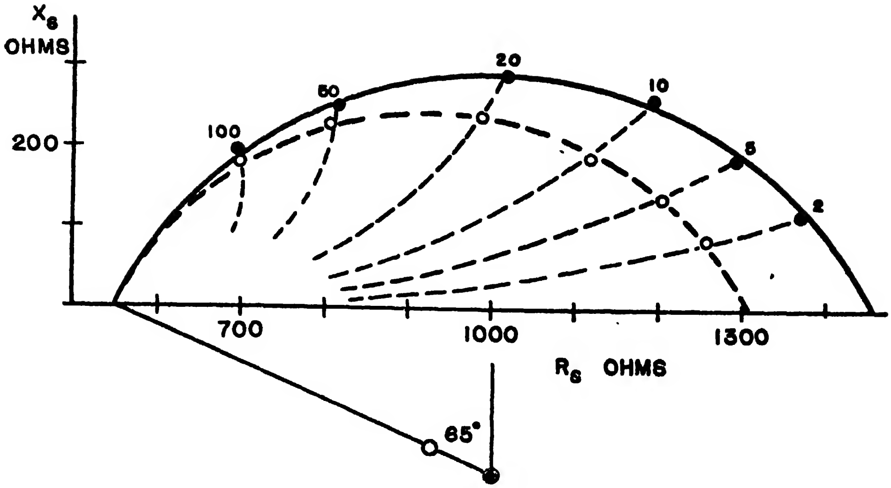

FIG. 7. Transverse impedance locus, series resistance, R_s, *vs.* series reactance, X_s, for axon at rest (●), and during cathode current flow of 125 μamp. (○). Frequencies are indicated in kilocycles. The light dotted lines represent the theoretical paths of the impedance at each frequency for a change of membrane conductance.

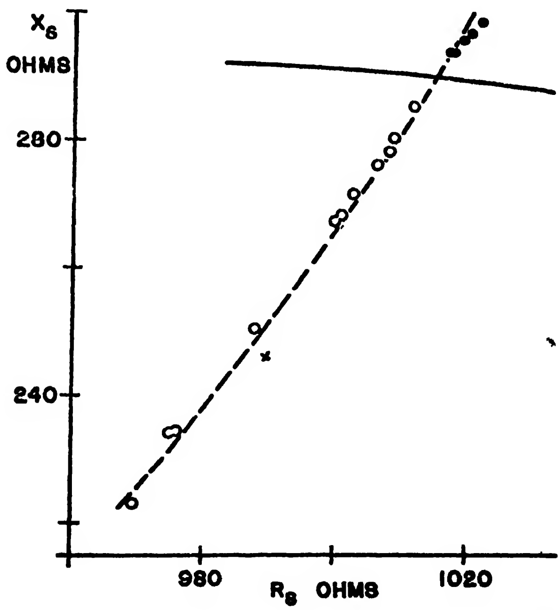

FIG. 8. Transverse impedance locus, series resistance, R_s, *vs.* series reactance, X_s, at 20 kc. during current flow. (○), Cathode currents up to 250 μamp., (●), anode currents up to 63 μamp. The solid line is portion of the locus for frequency variation and resting axon. The dotted line is a portion of the theoretical locus for constant frequency and a variation of the membrane conductance. (×), maximum impedance change during passage of a distantly initiated impulse.

polarizing current of 125 μamp. and three other axons give values for ΔG of 0.017, 0.026, and 0.033 ohm^{-1} cm.$^{-2}$

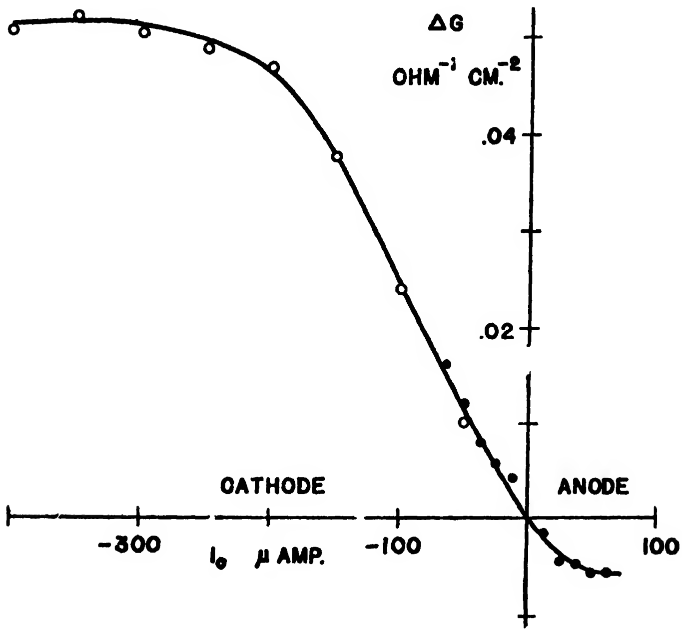

FIG. 9. Change of membrane conductance, ΔG_4, *vs.* total current flow, I_0, from two experiments on the same axon.

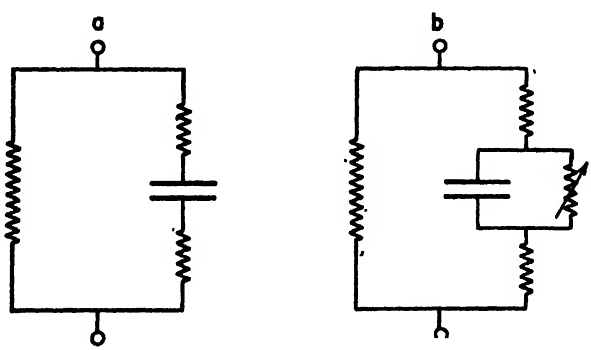

FIG. 10. Equivalent circuits for transverse impedance of axon. (*a*) Theoretical circuit neglecting membrane conductance. (*b*) Theoretical circuit with a variable membrane conductance depending upon current flow.

The variation of ΔG with polarizing current is usually obtained from data at a single frequency. The impedance for each value of the polarizing current may be extrapolated to zero frequency and the conductance

change calculated as above, but the impedance change is usually sufficiently small to be proportional to the conductance change (Cole and Curtis, 1939). The impedance change is then computed by

$$|\Delta Z| = \sqrt{(\Delta R_s)^2 + (\Delta X_s)^2}$$

or from photographic records, and $\Delta G = -K|\Delta Z|$, where the factor of proportionality, K, is obtained from one or two extrapolations. A curve of ΔG *vs.* I_0 (Fig. 9) shows values obtained by both methods and is typical of data on eleven axons.

DISCUSSION

To justify the interpretation of the observed impedance change on polarization as a change of membrane conductance, we proceed as before (Cole and Curtis, 1938). The circular path followed by the impedance of the resting fiber as the frequency is varied, seen in Fig. 7, and called the resting locus, is the characteristic result of many biological impedance measurements. On the assumption of a negligible membrane conductance we may calculate from theory (Cole and Curtis, 1936) the equivalent circuit shown in Fig. 10 *a*, where the condenser represents the capacity of the axon membrane. If the membrane capacity *alone* were to change, the impedance at each frequency would merely move along the resting locus; if the resistance of the axoplasm varied, only the infinite frequency extrapolation would be altered; and changing either the volume of the cell or the resistance of the sea water would vary both the infinite and zero frequency extrapolations. Since Figs. 7 and 8 do not allow any single one of these possibilities, we turn to a variation of the membrane conductance as indicated in Fig. 10 *b*. It has been shown (Cole and Curtis, 1938, 1939) that with a variation of this conductance alone, the impedance would follow a circular arc tangent to the resistance axis at the infinite frequency extrapolation. This is seen to be approximately true at each frequency in Fig. 7 and on an enlarged scale at 20 kc. in Fig. 8, it is found to represent the data to within the limits of experimental accuracy. We are thus entitled to conclude from these data that the impedance change during polarization may be interpreted as a change of the membrane conductance alone, and that there is an increase of the membrane conductance at the cathode and a decrease at the anode. When we assume that the membrane conductance is proportional to the ion permeability of the membrane, the results demonstrate an increase of permeability at the cathode and a decrease at the anode.

It will be noticed in Fig. 8 that the point of maximum impedance change

during the passage of a distantly initiated impulse is close to the theoretical locus but does not lie on it. This is the type of departure found in the previous work (Cole and Curtis, 1939) and was believed then to be caused by the amplifier characteristics. This hypothesis has been supported, although not yet proven, by further work, and even if it is not valid, the difference between this aspect of cathode polarization and propagated activity is very slight.

On the basis of the local circuit theory of excitation and propagation, at least a part of the membrane conductance increase during activity may be a result of membrane current flow, but in the previous discussions (Cole and Curtis, 1938, 1939) the implications of this possibility were avoided. Since a membrane conductance change is brought about by current flow and independently of excitation, it is now necessary to consider the method of measurement more carefully. As a result of a current flow, i, the potential difference across the membrane is altered in some way by an amount, v. When $v = Ri$ the membrane obeys Ohm's law and R is its resistance. The alternating-measuring current, i_a, and the direct-polarizing current, i_d, were applied simultaneously in these experiments and the resulting alteration of the potential difference across the membrane at any time depends upon the instantaneous sum of these two currents, so $v = f(i_d + i_a)$. If now the maximum value of the conduction component of the alternating current, i_a, is small compared to the direct current, i_d, we have by Taylor's expansion, approximately

$$v = v_d + v_a = f(i_d) + \frac{df(i_d)}{di_d} \cdot i_a$$

where the part of the potential difference caused by the polarizing current is v_d and by the measuring current is v_a. Since we are considering only the conduction component of the measuring current we have

$$v_a = \frac{df(i_d)}{di_d} \cdot i_a = \left(\frac{dv}{di}\right)_{i=i_d} \cdot i_a = r(i_d) \cdot i_a$$

where r is the "variational" resistance of the membrane as measured by a small alternating current. If now the membrane obeys Ohm's law, $v = ri$, we have $v_d = ri_d$; $v_a = ri_a$, and r is independent of the current. In graphical form, for Ohm's law, we have a straight line relation between the current and voltage and the variational resistance, or the slope of the line, remains unchanged for all values of current. In the present case, r, or the slope, depends upon the current, i, and Ohm's law certainly is not valid except as an approximation for the small variations of current which we have applied by the bridge.

Since we have now found that the membrane does not follow Ohm's law, some specifications of its conductive properties must be given in place of the resistance. The most obvious possibility is the potential difference as a function of the current which is obtained by the integration

$$v = f(i) = \int_0^i r(i)\,di,$$

for both anode and cathode. Our present data, however, only give changes of the membrane conductance such as Fig. 9 and it is necessary to have the resting conductance before the integration can be carried out. The value of 1000 ohm cm.^{2} obtained by other measurements (Cole and Hodgkin, 1939) could be used, but there is another difficulty. The polarizing membrane current density in the region between the impedance electrodes is also needed, and must be calculated from the total polarizing current. This may be done easily on the basis of the simple cable theory, if a constant membrane resistance can be assumed, but the process is quite tedious for the present problem where this assumption cannot be made. Since these results will be very dependent upon the value assumed for the resting conductance and the information may be obtained more directly from another type of experiment (Cole and Curtis, 1941), the calculations have not been carried out. However the general form of $f\,(i)$, or the v *vs.* i curve, is quite apparent. At the origin its first derivative, or slope, is given by the resting resistance, and on the anode side this slope increases with increasing current until a large limiting slope is reached. On the cathode side, the slope continually decreases until a small limiting slope is obtained for large currents. Then for a potential applied across the membrane with the anode outside, the current flow will be less than for the same potential with the cathode outside. It is then perfectly obvious that the axon membrane not only acts as a rectifier but probably also as a rather efficient rectifier.

Returning to the conductance change, it is seen that the maximum increase under the cathode (Figs. 8 and 9) is of the same order of magnitude as that found during the passage of an impulse. This conclusion may be of considerable significance, but it should not be emphasized at the present time because the cathode polarization currents necessary to give the maximum conductance change were used in only a few experiments. The maximum decrease of conductance at the anode in Fig. 9 is about 0.005 ohm^{-1} cm.$^{-2}$ and this is close to the average value found for four axons. If the resting membrane resistance were 200 ohm cm.^{2}, corresponding to a conductance of 0.005 ohm^{-1} cm.$^{-2}$, such a change would mean that the membrane becomes non-conducting under the anode and if the resting resistance

were greater than 200 ohm cm.^{2} and, consequently, the conductance less than 0.005 ohm^{-1} cm.$^{-2}$, the resistance calculated during current flow would be negative. Since we are not prepared to deal with the latter concept, we should conclude that the resting resistance was less than 200 ohm cm.^{2}. This is, however, much lower than the values of 400 to 1100 ohm cm.^{2} obtained by longitudinal measurements (Cole and Hodgkin, 1939), and no reason can be given for this discrepancy. The axons used for the transverse measurement were apparently in as good condition and survived at least as well as those used for the longitudinal measurements. The types of analysis used for the interpretation of the data are very dissimilar in the two cases, but no fundamental errors of assumptions or derivation have as yet been uncovered. It is felt, at present, that this disagreement is probably not a serious matter and that the results may be taken to indicate a very low limiting conductance under the anode.

On the basis of the present data, any discussion of the mechanisms of excitation and propagation is little more than speculation, but attention may be called to a few observations. One of these is the oscillation of the impedance change seen in Fig. 4 *a*, just below threshold. This is a characteristic of the responses at the cathode, down to about half threshold, and is not found at any anode polarization. There is then the further observation that the first maximum of this oscillation clearly becomes the all-or-nothing response at threshold (Fig. 4 *b*).

The change of membrane conductance has been determined as a function of the current in the steady state, but we have no evidence at present to indicate that this relation between current flow and membrane conductance remains unaltered during excitation and recovery. However, let us assume for the moment that with these data we may calculate the current flow through the rectifier element from the conductance change during the passage of an impulse. There is no change up to the point of inflection of the rising phase of the action potential. This corresponds to no current flow through the element and requires that the change of membrane potential difference occur elsewhere in the membrane. Such a conclusion seems reasonable and can probably be verified by a careful consideration of the conductance change after the application of a polarizing current. On the other hand, the increased conductance after the point of inflection would require an outward current flow in the rectifier element during all of the time that the total membrane current flow is inward and this will involve more detailed assumptions. Indeed from the steady state characteristics one would say that the only current flow through this element during the entire action would be outward, which would correspond to a net transport

of positive ions outward across the membrane. This is in the proper direction to recharge the membrane during the recovery phase and might lead us to conclude that the conductance increase is concerned primarily with recovery. Such a conclusion would agree with the observation that an axon nearly always failed to conduct soon after the impedance change during the passage of an impulse became too small to measure. However, these conclusions are not justified unless it can be shown that during excitation and recovery the dependence of the membrane conductance on current is the same as for a constant current flow.

SUMMARY

The change in the transverse impedance of the squid giant axon caused by direct current flow has been measured at frequencies from 1 kc. per second to 500 kc. per second. The impedance change is equivalent to an increase of membrane conductance at the cathode to a maximum value approximately the same as that obtained during activity and a decrease at the anode to a minimum not far from zero. There is no evidence of appreciable membrane capacity change in either case. It then follows that the membrane has the electrical characteristics of a rectifier. Interpreting the membrane conductance as a measure of ion permeability, this permeability is increased at the cathode and decreased at the anode.

BIBLIOGRAPHY

Cole, K. S., 1928, *J. Gen. Physiol.*, **12,** 29; 1932, **15,** 641.

Cole, K. S., and Cole, R. H., 1936, *J. Gen. Physiol.*, **19,** 609.

Cole, K. S., and Curtis, H. J., 1936, Electric impedance of nerve and muscle, in Cold Spring Harbor symposia on quantitative biology, Cold Spring Harbor, Long Island Biological Association, **4,** 73. 1937, *Rev. Scient. Instr.*, **8,** 333. 1938, *J. Gen. Physiol.*, **22,** 37; 1939, **22,** 649; 1941, **24,** 55.

Cole, K. S., and Hodgkin, A. L., 1939, *J. Gen. Physiol.*, **22,** 671.

Young, J. Z., 1936, Structure of nerve fibers and synapses in some invertebrates, in Cold Spring Harbor symposia on quantitative biology, Cold Spring Harbor, Long Island Biological Association, **4,** 1. 1939, *Phil. Tr. Roy. Soc. London, Series B*, **229,** 465.

MEMBRANE POTENTIAL OF THE SQUID GIANT AXON DURING CURRENT FLOW

By KENNETH S. COLE AND HOWARD J. CURTIS

(From the Department of Physiology, College of Physicians and Surgeons, Columbia University, New York, and The Marine Biological Laboratory, Woods Hole, Massachusetts)

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INTRODUCTION

At the time the experiments on the impedance change of the squid giant axon during current flow (Cole and Baker, 1941) were planned, it was realized that the measurement of the change of membrane potential during current flow was equally important. From the transverse impedance experiments it was possible to eliminate variations of the internal and external resistances, axon volume, and the membrane capacity and to express the change entirely as one of membrane conductance. An increase of membrane conductance could be determined satisfactorily from the measurements, but a decrease could only be approximated and the resting reference level was out of the question (Curtis and Cole, 1938). There was the further possibility that a critical change of membrane electromotive force without an appreciable change of conductance might go unnoticed. On the other hand, the change of membrane potential during current flow should give an independent measurement of the resting membrane resistance and capacity as well as changes of potential. These measurements should be particularly satisfactory for a decrease of membrane conductance, but they might not be so useful for an increase or for small changes of conductance. Although it is to be expected that the potential and impedance changes can be correlated ultimately, the membrane potential measurements are at the present time more closely associated with the extensive external potential measurements which have been made on many other nerves.

In the past, it has been possible to make potential measurements with external electrodes only, and it is an indirect and rather uncertain procedure to estimate the membrane potential from these (Cole and Curtis, 1939). With the introduction of Young's giant axon preparation from the squid (Young, 1936) and the capillary electrode technique (Hodgkin and Huxley, 1939; Curtis and Cole, 1940) it has become possible to make direct measure-

ments of the membrane potential. The present experiments were undertaken primarily to investigate the relation between the steady state change of membrane potential and the membrane current and also to determine whether or not, with high cathode polarizations, the potential was the same before and after excitation (*cf.* Cole and Baker, 1941). It was also planned to analyze the transients at the make and break of the polarizing current as completely as possible.

Material and Apparatus

The giant axon from the hindmost stellar nerve of the Atlantic squid *Loligo pealii* was dissected out and teased free from small fibers. It was then placed in a transverse impedance cell consisting of a sheet of insulating material in the top of which was cut a trough about 500μ square and just large enough to accommodate the axon. Square platinized lead impedance electrodes were set flush with the sides of the trough and opposite each other. A thin glass cover slip was placed over the top of the cell after the axon was in place. As before (Cole and Baker, 1941), the polarizing current was applied by the cam contactor to the two impedance electrodes in parallel and to a remote electrode at one end of the cell. Resistances of from $1.5 \cdot 10^4$ to $5 \cdot 10^5$ ohms in series with the battery maintained approximately constant current as shown in Fig. 1.

At first the external potential was measured between one of the impedance electrodes and an electrode at the other end of the trough relative to the remote polarizing electrode. Although the alternating current impedance of a platinized electrode was only slightly affected by the current flow, the polarization potential of the electrode practically obscured the small potential change of the axon membrane. The capillary needle technique (Curtis and Cole, 1940) was then applied and the potential difference measured between an impedance electrode and the capillary of the needle whose tip was in the axoplasm midway between the two impedance electrodes. This also was unsuccessful because the electrode polarization practically obscured even this larger potential. An outside potential electrode was then constructed by imbedding a fine glass capillary in the top surface of the cell with its tip at the center of the grounded impedance electrode and flush with its surface. This capillary was filled with sea water and an electrode was fixed at the opposite end. Completely satisfactory potential measurements could now be made between the inside and outside needles with the polarizing current applied as before. When the polarizing current was applied to the cell filled with sea water, but without an axon, a potential was obtained under some conditions, having a maximum value about 10 per cent of that obtained with the axon. This effect was not investigated in detail, but corrections were made where it was measurable. The amplifier and cathode ray oscillograph have been described (Cole and Curtis, 1939).

EXPERIMENTAL

Records were first taken of the membrane potential as a function of time for a series of polarizing currents. The membrane potentials for anode and cathode polarizing currents of 9.7, 24, and 48 μamp. are shown in Fig. 2 *a*, *b*, and *c* respectively. At the lowest value of polarizing current, which

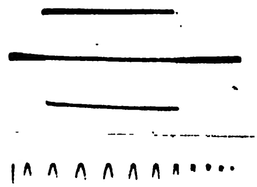

FIG. 1. Oscillograph records of cathode and anode current flow and base line made on three successive sweeps. Exponential sweep, timing, 200 cycles.

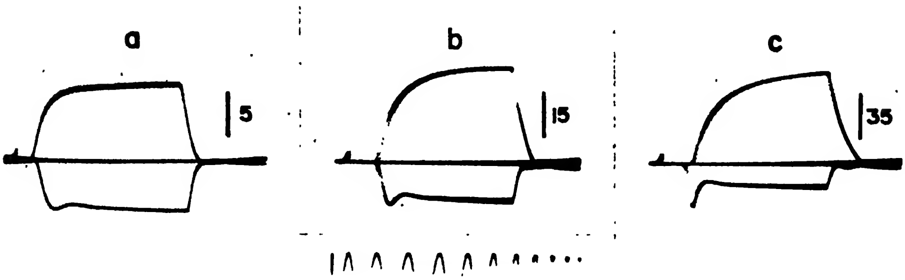

FIG. 2. Oscillograph records of change of membrane potential during current flow with the resting potential as base line, made on three successive sweeps in each case.

Anodes upward, and cathodes downward, are, respectively, increases and decreases of the membrane potential. Total current flow in μamp., (*a*) 9.7; (*b*) 23.7 (below rheobase); (*c*) 47.5 (above rheobase). Potential calibrations indicated are in millivolts. Exponential sweep, timing, 500 cycles.

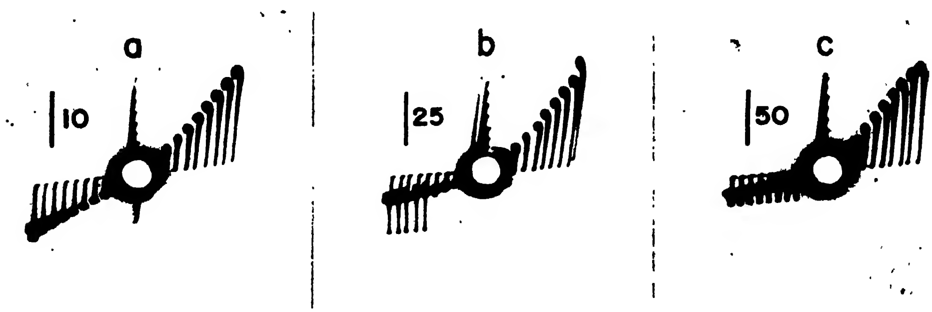

FIG. 3. Oscillograph records of change of membrane potential (ordinates) *vs*. approximate total current flow (abscissae), anode up and to right, cathode down and to left. Maximum values of current in μamp. are (*a*) 17.5; (*b*) 47.5; (*c*) 95. Current steps are each 10 per cent of the maximum. Potential calibrations indicated are in millivolts.

The dark spots corresponding to each value of current indicate the steady state change of membrane potential caused by the current. The excursions of the potential change below these steady state values on the cathodal side in *b*) and *c*) are the action potentials initiated by the make of the larger currents

is about 0.4 rheobase, the difference between anode and cathode is seen both in the behavior of the potential at the start of the current and in the constant level of potential finally reached. Although the initial rate of rise of potential is approximately the same for both anode and cathode there is a distinct oscillation at the cathode which is not seen at the anode and the final value of potential at the cathode is somewhat lower than at the anode. When the current is increased to 24 μamp. (Fig. 2 *b*) barely sub-rheobasic, the cathode oscillation has somewhat greater amplitude, the anode rise is considerably slower, and the discrepancy between the final potentials is larger. In going to 48 μamp. (Fig. 2 *c*) nearly twice rheobase, the first maximum at the cathode has become the propagated impulse in the characteristic all-or-nothing manner. The establishment of the steady anode potential is even slower and the ratio of final potentials is still further increased.

Since there are obviously a number of factors involved in the initial or transient behavior of the potential, attention was first centered on the steady state characteristics. It then became convenient to record as much of the information as possible on a single film, and this was done by removing the sweep circuit voltage from the horizontal deflecting plates of the oscillograph and replacing it by a potential, derived from the polarizing circuit, and proportional to the polarizing current. When the current was applied (Fig. 3) the oscillograph spot gave a sudden horizontal deflection from the center point proportional to the current, and then moved vertically as the membrane potential developed as was seen in Fig. 2. At the cessation of the current, the spot returned suddenly to the vertical axis, and then descended to the center more slowly as the potential returned to its resting value. The polarizing current was applied at intervals of about 1 second and by changing the current during the off period of each interval the complete current-potential series of Fig. 3 *a*, *b*, or *c*, could be obtained in about 20 seconds. In Fig. 3 *a*, the maximum current of 17.5 μamp. was sub-rheobasic but the oscillations of potential at the cathode are apparent in the width of the spot at the higher currents. A decided departure from a linear relation between current and potential is quite evident. As we go to the maximum value of 47.5 μamp. (Fig. 3 *b*) the rheobase was exceeded and the five highest values of current gave rise to propagated impulses with the potential falling considerably below the steady value, and the curvature of the locus of the steady values is even more marked. For a maximum value of 95 μamp. (Fig. 3 *c*) there are eight points above threshold and the current-potential relations at the anode and cathode are very striking. The complete data on one axon after

correction and reduction to common potential and current scales have been plotted in Fig. 4.

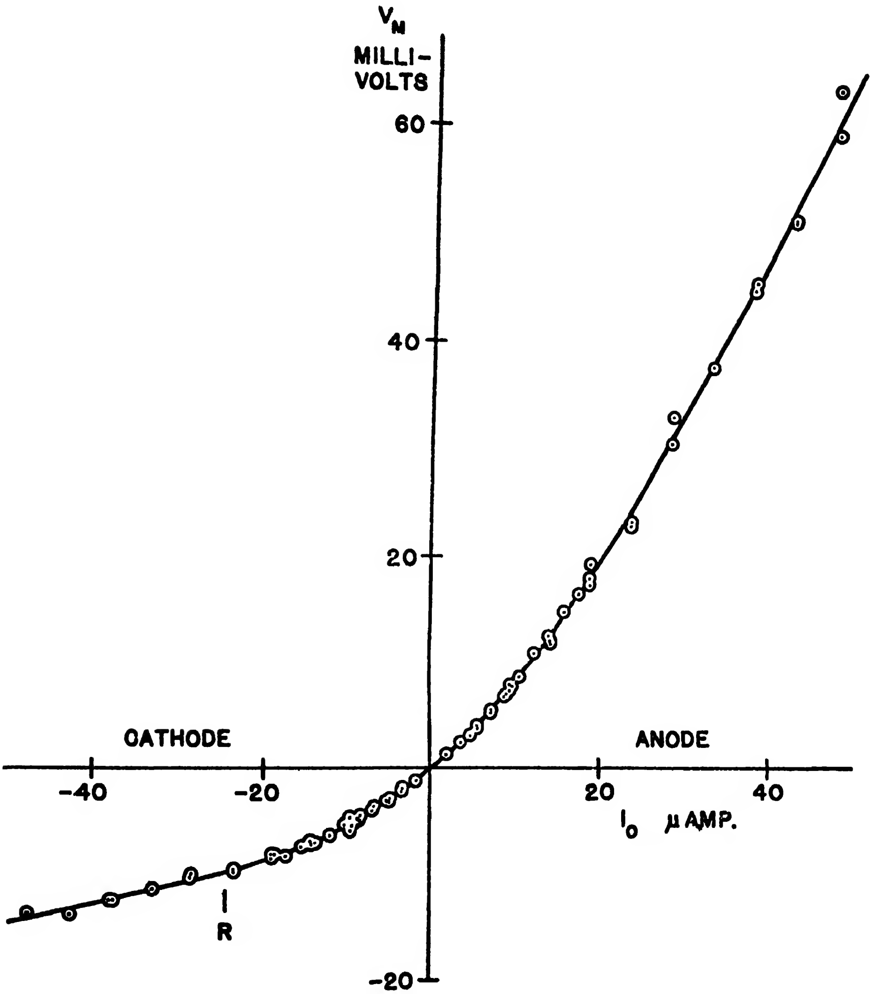

FIG. 4. Change of steady state membrane potential, V_M, *vs.* total current flow, I_0. Composite curve showing data of five experiments on the same axon. Rheobase indicated by *R*.

As the threshold for excitation rose and the fiber finally became inexcitable, the polarizing current necessary for a given change of membrane potential increased, and the potentials under the anode and cathode became more and more nearly equal to each other.

At the start of the experiments on the impedance change during current

flow it was anticipated that a large polarizing current might maintain a depolarization after an excitation. No evidence for this was found and a similar conclusion is to be drawn from the present experiments. Experiments on the potential have been performed with sub-threshold exponentially rising currents which gave the same steady state characteristic as when excitation took place at a sudden make. In Fig. 3 *b* and *c*, it is apparent that the steady state points form a continuous smooth curve up to four times rheobase and from Fig. 4 there is no certain change of form as rheobase is passed. It was also found that after a steady membrane potential had been reached in the polarized region, either anodic or cathodic, the potential returned to this same value after a distantly initiated impulse had passed through the region.

Calculations

The first parameter to be computed is the resting membrane resistance This may be obtained from the curves for the change of membrane potential V_M, *vs.* the polarizing current, I_0, such as the one shown in Fig. 4. For sufficiently small polarizing currents, *i.e.* near the origin, these curves may be approximated by straight lines, and the slope, $V_M/I_0 = \bar{R}$, has the dimensions of a resistance. In this region of 1 or 2 microamperes, we are entitled to treat the membrane in the steady state as a resistance, r_4, for a unit length of axon, and the usual cable equation may be applied.

When r_1, r_2 are the resistances for a unit length outside and inside the axon membrane, and i_1, i_2 are the corresponding currents, it is found (Cole and Hodgkin, 1939, equation 11) that in the interpolar region (*i.e.* between the polarizing electrodes), at a distance x from the midpoint between the electrodes, the membrane potential difference is $V_M = (r_1 i_1 - r_2 i_2)\lambda \tanh (x/\lambda)$, where $\lambda = \sqrt{r_4/(r_1 + r_2)}$. Then if x is large, this approaches

$$V_M = (r_1 i_1 - r_2 i_2)\lambda. \tag{1}$$

By a similar procedure it is found that in an extrapolar region, *i.e.* outside the polarizing electrodes, and at a considerable distance from the origin at the end of the axon, we have again

$$V'_M = -(r_1 i'_1 - r_2 i'_2)\lambda. \tag{2}$$

For a narrow electrode—about as wide as the diameter of the axon—the membrane potential under it may be considered also as the interpolar and extrapolar membrane potential, $V_M = V'_M$. On the interpolar side,

$i_1 + i_2 = I_0$ and on the extrapolar, $i_1' + i_2' = 0$, while the interpolar and extrapolar inside currents are obviously equal, $i_2 = i_2'$. Then from (1) and (2)

$$i_2 = \frac{r_1}{2(r_1 + r_2)} I_0 \quad \text{and by (1),} \quad V_M = \frac{r_1 \lambda}{2} I_0. \tag{3}$$

So

$$\frac{V_M}{I_0} = \bar{R} = \frac{r_1 \lambda}{2} \quad \text{and} \quad r_4 = \frac{4(r_1 + r_2)}{r_1^2} \bar{R}^2 \tag{4}$$

As representative values we may take $r_1 = 1.9 \cdot 10^4$ ohm/cm., $r_2 = 1.8 \cdot 10^4$ ohm/cm. to compute r_4 by equation (4), then for a membrane

TABLE I

$\bar{R}$ ohms	r_4 ohm cm.	R_4 ohm cm.^{2}
500	100	14.4
770	240	34.
540	120	17.
640	170	23.5
840	290	40.5
(375)	(57)	(8.1)
660	180	25.
		23. Average

area per unit length of 0.14 cm.^{2}/cm. we obtain the membrane resistance, R_4, for a square centimeter. These values are given in Table I from the available data. The values in parentheses are for an inexcitable axon which was excluded from the average.

When the polarizing current exceeds more than a few per cent of the rheobase, it is no longer permissible to consider the membrane conductance as a resistance, r_4. Since as yet there is no evidence that the external and internal media may not be considered as resistances, r_1 and r_2, we may use the cable equation (Cole and Curtis, 1938, equation 10) in the form

$$\frac{d^2 V_M}{dx^2} = (r_1 + r_2) I_M,$$

where I_M, the membrane current density, is now to be determined as a function of V_M. At a sufficient distance from the electrode in the extrapolar region there is neither an appreciable polarizing current flow across

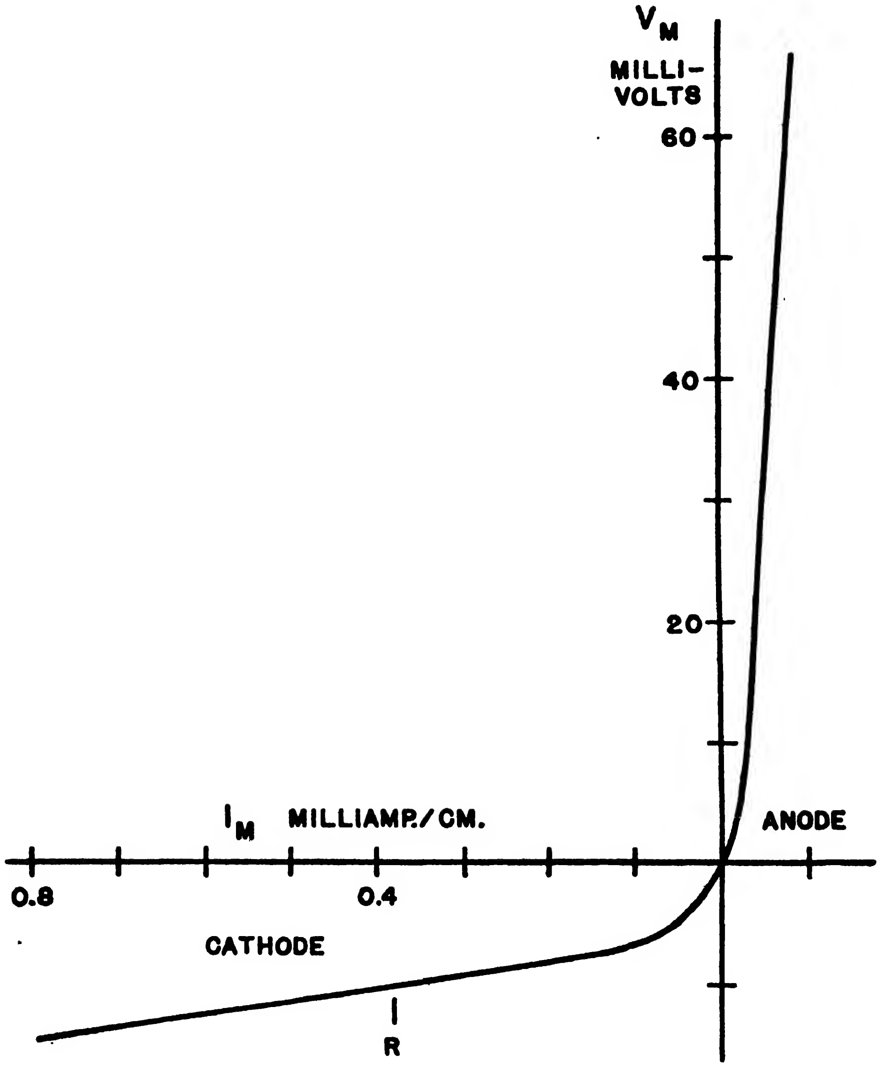

FIG. 5. Change of steady state membrane potential, V_M, *vs.* membrane current density, I_M, as calculated from data of Fig. 4 by equation (6). Rheobase indicated by R.

the membrane nor a potential difference caused by it. As we approach a point at a distance x from the electrode, we have at each point,

$$\frac{dV_M}{dx} = (r_1 + r_2)i_1'(x)$$

and

$$\frac{d^2V_M}{dx^2} = (r_1 + r_2)\frac{di_1'}{dx} = (r_1 + r_2)^2 i_1' \cdot \frac{di_1'}{dV_M};$$

then

$$I_M = (r_1 + r_2)i_1' \cdot \frac{di_1'}{dV_M}. \tag{5}$$

Then at the electrode, by equation (3)

$$i_1' = -\frac{r_1}{2(r_1 + r_2)} I_0$$

and we find from equation (5) that

$$I_M = \frac{r_1^2}{4(r_1 + r_2)} \cdot I_0 \cdot \frac{dI_0}{dV_M} \tag{6}$$

When dI_0/dV_M is independent of I_0 this reduces to the previous case, equation (4). We have I_0 and may determine dI_0/dV_M from Fig. 4 for each value of V_M. The membrane current densities, I_M, then found by equation (6) and the membrane potential, V_M, have been plotted in Fig. 5.

The "variational" conductance, $G_4 = dI_M/dV_M$ may now be taken directly from Fig. 5. This has been plotted against the total polarizing current, I_0, in Fig. 6 for comparison with similar data from the impedance change during current flow (Cole and Baker, 1941, Fig. 9).

DISCUSSION

Although the impaled axons often survived for several hours at room temperature and without circulating sea water, and the data could be taken with considerable rapidity, the reproducibility of the measurements shown in Fig. 4 was both surprising and gratifying.

The wide discrepancy between the present average value of 23 ohm cm.^{2} for the resting membrane resistance and the average value of 700 ohm cm.^{2} found by Cole and Hodgkin (1939) calls for some comment. There are many possible factors because the two experiments have little in common except that they are both direct current measurements on the squid axon. The first factor, which at the present time seems to be the most important, is the physiological condition of the axon. For the longitudinal resistance experiment the axon had to have a high membrane resistance,—otherwise the characteristic length would be so short that measurements could not be made with sufficient accuracy. It was also found that the high resistance correlated rather well with good physiological condition and survival. In the transverse impedance work, it was found that the impedance change during activity was also a rather sensitive index of the condition of the axon, more so than the action potential, for example. Consequently we might expect that a high membrane resistance would be

associated with a large impedance change during activity and *vice versa*. On this basis we should not be surprised at the low membrane resistance now found, because as has been pointed out (Curtis and Cole, 1940) the impedance change during activity was considerably lower immediately after impalement than it had been before. Furthermore, the most complete data, such as in Figs. 3 and 4, were obtained on axons for which the action potential had the average value of 50 mv. found by Curtis and Cole (1940). This was considerably below the maximum potentials obtained on axons in better condition. From this point of view, it seems possible that both sets of measurements may be essentially correct, and that they represent the membrane resistances of axons in different physiological conditions.

The other principal factor to be considered is the calculation of the results. In the longitudinal measurements with infinite electrodes the theory is relatively straight forward and represents the experimental conditions quite well, and furthermore, sufficient data could be taken to furnish a rather satisfactory check on the form of the theoretical expression. In the present case, however, the geometry is much more complicated and an exact solution is out of the question. The only simple approximations are those of a small axon diameter and a negligible electrode length at the point where measurements were made. An electrode length of 0.5 mm. is not negligible, but it is, after all, approximately the axon diameter. One approximation is then as good or as bad as the other and each will have to stand until both can be improved upon. This state of affairs is even more unfortunate because no reasonable experiments have been found which can either prove or disprove the validity of the equations used for calculation. Another difficulty is that direct measurements of the internal and external resistances, r_1 and r_2, could not be made, and the estimates used in the calculations may not be particularly good. As will be seen in equation (4), relatively small errors of r_1 have a large effect on the calculated membrane resistance, r_4.

The transverse impedance during current flow gave the change of membrane conductance with the total polarizing current (Cole and Baker, 1941, Fig. 8). This may be compared with the variational membrane conductance as a function of the total polarizing current obtained in the present experiments (Fig. 6). These two curves should be the same, except that a resting membrane conductance may be obtained from the present data, and an obvious similarity between them is found. Each approaches a constant value of conductance at both high anode and high cathode polarizations, and in each the change of this asymptotic value from that at rest is about ten times as large at the cathode as at the anode. The absolute

values of the conductances and of the polarizing currents are, however, considerably different. Although the two sets of measurements were not made on the same axon nor in the same measuring cell, these two measuring cells were so nearly identical that no serious difference in the flow of the polarizing current would be expected. An appraisal of the analysis used for the interpretation of the data in each experiment is again a difficult

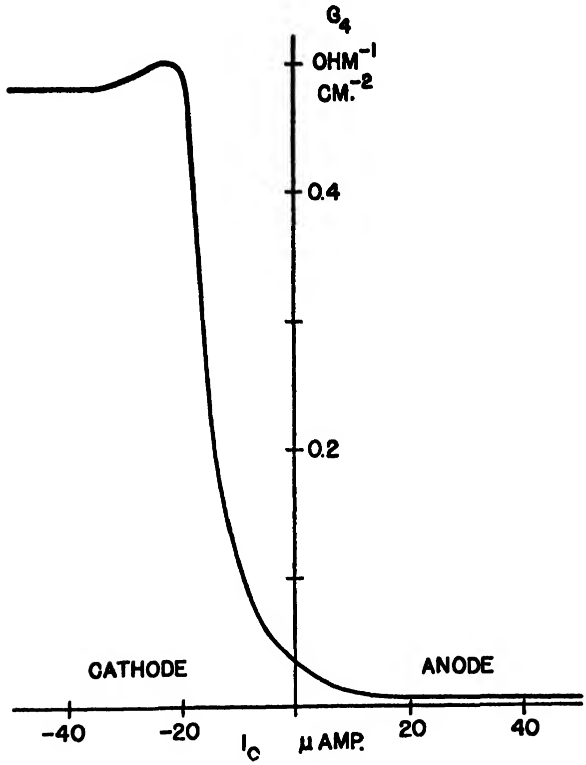

FIG. 6. Calculated membrane conductance under electrode, G_4, *vs.* total current flow, I_0.

task. As we have seen, there are a number of unsatisfactory aspects to the potential measurements, and the results of the impedance experiments also involve a number of compromises between theory and experiment which are not easily evaluated. Consequently, until the discrepancies in the magnitudes of the membrane conductance and polarizing current can be definitely ascribed to the measurements or their analysis, it is not reasonable to assume that all the differences were actually in the axons. Yet, as has been pointed out above, the most probable single factor is the puncture of the axon and its rather immediate consequences. In view of this, it may

be more appropriate at the present time to overlook the differences and consider the common characteristics of the data and analyses of the two types of experiments as good evidence in support of each.

The performance of rectifiers has been expressed in various forms depending upon the use to which the information was to be put, but the most convenient specification of the rectification characteristics of the membrane is from either the variational resistances or the conductances at rest and at the maximum and minimum values. It is found (Fig. 6) that at the cathode the conductance is about thirteen times that at rest, while at the anode it is about one-eighth the conductance at rest. Thus the ratio of the maximum conductances in the two directions is greater than one hundred to one and this is representative of the other data obtained.

It should be possible to calculate the membrane capacity from the initial rate of rise of the potential in Fig. 2 but before this could be done it was necessary to consider the amplifier characteristics. The combination of the needle electrode resistance and the input capacity of the amplifier was found subsequently to be the controlling factor which precluded the use of these data. The oscillations which appear farther along in the initial transient of the membrane potential under the cathode are particularly interesting. They agree in a general way with those found in the impedance change under similar conditions, for the frequency and amplitude are similar and the propagated all-or-none response again appears at the first maximum. Although a detailed analysis would probably be misleading because of the apparatus limitations, the oscillatory response seems quite certain, and its appearance in both types of measurement emphasizes their common basis.

SUMMARY

The squid giant axon was placed in a shallow narrow trough and current was sent in at two electrodes in opposite sides of the trough and out at a third electrode several centimeters away. The potential difference across the membrane was measured between an inside fine capillary electrode with its tip in the axoplasm between the pair of polarizing electrodes, and an outside capillary electrode with its tip flush with the surface of one polarizing electrode.

The initial transient was roughly exponential at the anode make and damped oscillatory at the sub-threshold cathode make with the action potential arising from the first maximum when threshold was reached.

The constant change of membrane potential, after the initial transient,

was measured as a function of the total polarizing current and from these data the membrane potential is obtained as a function of the membrane current density. The absolute value of the resting membrane resistance approached at low polarizing currents is about 23 ohm cm.^{2}. This low value is considered to be a result of the puncture of the axon. The membrane was found to be an excellent rectifier with a ratio of about one hundred between the high resistance at the anode and the low resistance at the cathode for the current range investigated.

On the assumption that the membrane conductance is a measure of its ion permeability, these experiments show an increase of ion permeability under a cathode and a decrease under an anode.

BIBLIOGRAPHY

Cole, K. S., and Baker, R. F., 1941, *J. Gen. Physiol.*, **24**, 535.

Cole, K. S., and Curtis, H. J., 1938, *J. Gen. Physiol.*, **22**, 37; 1939, **22**, 649.

Cole, K. S., and Hodgkin, A. L., 1939, *J. Gen. Physiol.*, **22**, 671.

Curtis, H. J., and Cole, K. S., 1938, *J. Gen. Physiol.*, **21**, 757; 1940, *J. Cell. and Comp. Physiol.*, **15**, 147.

Hodgkin, A. L., and Huxley, A. L., 1939, *Nature*, **144**, 710.

Young, J. Z., 1936, Structure of nerve fibers and synapses in some invertebrates, in Cold Spring Harbor symposia on quantitative biology, Cold Spring Harbor, Long Island Biological Association, **4**, 1. 1939, *Phil. Tr. Roy. Soc. London, Series B*, **229**, 465.

THE CHLOROPHYLL-PROTEIN COMPOUND OF THE GREEN LEAF*

By EMIL L. SMITH**

(From the Molteno Institute, University of Cambridge, England, and the Laboratory of Biophysics, Columbia University, New York)

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I

INTRODUCTION

Until 1870 it was assumed that chlorophyll extracted from the leaf by alcohol, acetone, or similar solvents, was the same as the green pigment in the leaf. In that year, Hagenbach found that the red absorption band of the leaf was 10 to 20 mμ further towards the red end of the spectrum than the corresponding band in the extracts. He later (1874) noted that the maximum of the weak leaf fluorescence was displaced in the same way with respect to the strong fluorescence of chlorophyll in solution. These observations have been repeatedly confirmed (*e.g.*, Hubert, 1935; Dhéré, 1937), and additional differences between the leaf pigment and chlorophyll solutions have since been observed, particularly with regard to solubility and photostability.

Among the many suggestions that have been offered to explain these differences are that the leaf pigment is dispersed in (Tschirch, 1883) or combined with lipoid (Palladin, 1910); that the pigment is colloidally dispersed (Herlitzka, 1912) and possibly adsorbed as a monomolecular layer on protein (Willstätter and Stoll, 1913; Noack, 1927).

In recent years, under the influence of the progress in the study of the respiratory proteins and enzymes, there has been a steadily growing notion that leaf chlorophyll is combined with protein (Lubimenko, 1927; Osborne, 1928; Mestre, 1930; Hubert, 1935; Stoll, 1936; Smith, 1938). Nevertheless, only little evidence has been forthcoming to prove this viewpoint. It is our intention to show that the properties of the green leaf pigment are best explained in terms of a true stoichiometric combination of chlorophyll with

* Short notes on this work have already appeared (Smith, 1938; 1940).

** John Simon Guggenheim Memorial Fellow (1938–1940).

protein, and to describe some of the properties of this compound.[1] The studies of French (1938, 1940) have demonstrated that the chlorophyll of photosynthetic bacteria is also bound to protein, showing that this linkage is general in nature.

II

Most of the observations were made with a simple direct extract of the leaves of spinach (*Spinacia oleracia*). Leaves were separated from stems, washed thoroughly, and then ground mechanically in a porcelain mortar with sand and a neutral or slightly alkaline buffer solution. The sand and cell debris were removed by centrifuging at low speeds. Opaque dark green preparations were obtained which show the dull red fluorescence characteristic of the leaf.

Both Osborne and Wakeman (1920), and Noack noted that such crude leaf extracts from spinach show extremely fine particles or globules under the microscope, so that what was actually studied was a suspension of the chloroplast material. The suspended chloroplast material can be separated in a variety of ways. It is sedimented by centrifuging at moderate speeds (3000-4000 R.P.M.), only a yellow or brown supernatant fluid remaining. It can also be separated by filtration through a thick layer of paper pulp or a Seitz bacterial filter, or by filtering through kieselguhr or Celite. All these separations indicate that the chloroplast material is not in a molecularly dispersed solution. The insolubility of the chlorophyll-protein complex appears to be due to the hydrophobic character of the chlorophyll, and the other lipoids associated with it in the chloroplast. This is indicated by the work of Menke (1938), who found that 37 per cent of the dry weight of the chloroplast (including the chlorophyll) is soluble in alcohol and ether.

Some extracts were made from the leaves of *Aspidistra lurida* because it was reported by Lubimenko that this species gives aqueous extracts which are completely water-clear and that the green pigment is in true aqueous solution. We have been unable to confirm this observation While the *Aspidistra* extracts appeared somewhat clearer than those from the spinach leaf, the extracts were always strongly opalescent. For purposes of comparison, most of the observations in this paper were made with the leaves of both species. Unless specific differences are indicated, observations may be taken to apply to both species.

III

Colloidal Chlorophyll

The characterization of the leaf pigment has depended in large part on the position of its absorption bands. Considerable controversy has attended efforts to explain the position of the red absorption band of the leaf on the basis of Kundt's rule. Mestre has summarized the evidence which

[1] At the moment it seems preferable to leave open the question of a name for this compound. It has been pointed out to us that the term "phyllochlorin" suggested by Mestre which we used in an earlier paper applies to a specific chemical derivative of chlorophyll. Other names which have been suggested are "chlorophylle naturelle" (Lubimenko), "chloroplastin" (Stoll), and "photosynthin" (French).

effectively disposes of this suggestion. However, many insoluble pigments show a shift in their band positions depending on the degree of dispersion. A striking example in a naturally occurring pigment is turacin, the copper-porphyrin compound of turaco feathers (Keilin, 1926). In order to show that such factors were not concerned, a comparison was made between the leaf pigment and colloidal chlorophyll in various states of dispersion. The spectral observations were confined to the region between 520 and 700 mμ since the absorption of other leaf pigments can be neglected in this region.

Herlitzka and later Willstätter and Stoll believed that the leaf pigment was colloidal chlorophyll, mainly on the basis of the similarity in position of the main red absorption band. Ivanovski (1907, 1913) opposed this view on the ground that not only were the band positions slightly different, but that the relative intensities of the various bands were different. Hubert found recently that the main red band of the leaf was at 680–681 mμ while that of colloidal chlorophyll was always further towards the blue but depended on the state of aggregation.

Colloidal chlorophyll was prepared by rapidly diluting crude acetone extracts of the leaf with a slightly alkaline phosphate buffer in order to prevent phaeophytin formation. The colloidal chlorophyll was dialyzed in cellophane membranes in the refrigerator against phosphate buffer in order to remove the acetone completely. The maximum absorption was always found in the region between 671 and 673 mμ as measured with a Hilger-Nutting spectrophotometer. The maximum absorption of aqueous leaf extracts was consistently at 677–678 mμ. Attempts were made to duplicate the appearance of the leaf pigment by preparing colloidal chlorophyll in the presence of proteins such as gelatin and horse serum. In every instance the red absorption maximum was the same as in the ordinary colloidal chlorophyll preparations.

Preparations of colloidal chlorophyll can be clarified, removing the characteristic bluish opalescence by adding a detergent such as digitonin or bile salts. The band position was then found to shift to 674–675 mμ. The shift towards the red can be explained by the removal of the light scattering, since the amount of scattering is proportional to the reciprocal of the fourth power of the wave length according to the Raleigh equation.

Differences in the positions of the absorption band in the red are always apparent; for colloidal chlorophyll the band is at 671–673, for the aqueous leaf extract at 677–678, and for the leaf itself at 681 mμ (Hubert). Ivanovski's observations are confirmed not only on this point, but also on the fact that the relative intensities of the absorption bands are different; the minor absorption bands *circa* 540 and 580 mμ like those of chlorophyll in organic

solvents are always much more prominent in colloidal chlorophyll than in the leaf or its aqueous extracts. This is likewise true for the main red bands of chlorophylls *a* and *b*; the separate *b* band is more prominent in colloidal chlorophyll or in organic solvents than it is in the leaf. These differences are very striking when spectra of the different preparations are observed side by side with a low dispersion spectroscope.

No fluorescence was observed with colloidal chlorophyll preparations confirming the older observations of Noack. Meyer (1939) has claimed that preparations of colloidal chlorophyll do fluoresce. We have made similar observations when relatively large amounts of alcohol or acetone were present; after removal of the organic solvent by dialysis, no fluorescence could be observed.

IV

Some Properties of the Leaf Pigment

As yet no specific catalytic property of the chlorophyll-protein has been observed. In order to characterize the material, it has been studied under various conditions.

Absorption Spectrum.—The absorption spectrum of an aqueous extract of spinach is given in Fig. 1; the data are presented in Table I. The measurements were made with the photoelectric spectrophotometer of Shlaer (1938). Absolute extinction values cannot be given for the unpurified extract because of the presence of various yellow substances (blue-absorbing), and because of the light-scattering produced by the suspended particles. This latter effect is clearly shown by the apparent absorption between 700 and 750 mμ. With an *Aspidistra* extract of comparable concentration (same extinction at 677 mμ) there is nearly the same amount of scattering in this region indicating a similar state of dispersion for the *Aspidistra* and spinach proteins.

The maximum absorption at the red end of the spectrum has always been found at 677 to 678 mμ. Secondary bands are at 625 and 590, with a definite inflection at 650 mμ. The minimum absorption is at 560 mμ. The absorption bands in the short wave region are at 470 and 437. These latter bands are the resultant not only of chlorophylls *a* and *b* but of the carotenoids as well. With *Aspidistra* extracts it is frequently possible, using a low dispersion microspectroscope, to separate two absorption bands, one at 470 and the other at 485–490.

Various substances have been tested for possible effect on the absorption spectrum of leaf extracts either because of their influence on photosynthesis or because they combine with some chromoproteins which are involved in

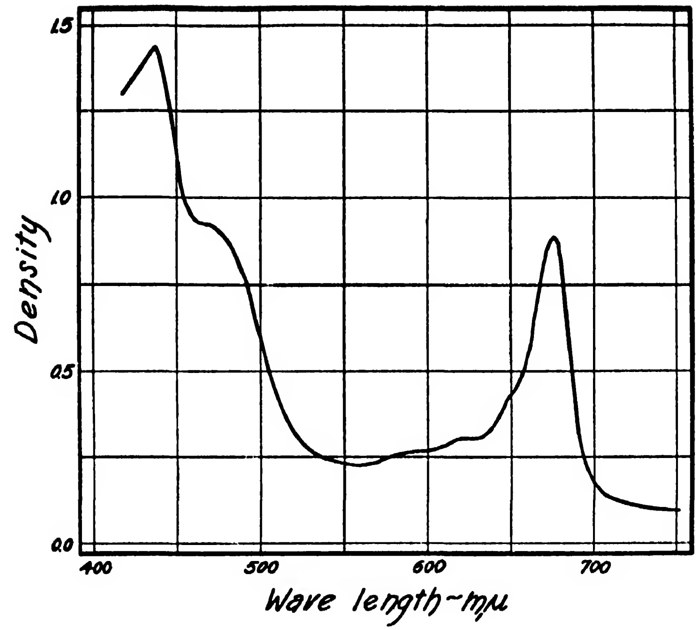

FIG. 1. Absorption spectrum of an aqueous extract from the spinach leaf. The data are given in Table I.

TABLE I

Absorption Spectrum of Leaf Extract

Data for an aqueous extract of spinach leaves buffered at pH 7.0 with 0.1 M phosphate buffer.

λ	Density	λ	Density	λ	Density
mμ		mμ		mμ	
750	0.0914	660	0.5224	530	0.2628
740	0.0958	650	0.4282	520	0.3132
730	0.1022	640	0.3382	510	0.4172
720	0.1116	630	0.3014	500	0.5878
710	0.1296	625	0.3026	490	0.7816
700	0.1774	620	0.3002	480	0.8608
695	0.2358	615	0.2908	475	0.9024
690	0.3602	610	0.2800	470	0.9176
685	0.5954	600	0.2672	465	0.9216
680	0.8454	590	0.2622	460	0.9376
678	0.8780	580	0.2516	450	1.0928
677	0.8860	570	0.2362	440	1.3928
676	0.8820	560	0.2246	435	1.4312
675	0.8766	555	0.2260	430	1.3848
670	0.7962	550	0.2304	420	1.3184
665	0.6594	540	0.2428		

tissue respiration. The tests were made by evacuating a control solution in a Thunberg tube and comparing it side by side with the test sample under the microspectroscope. Among the substances which have been tested are: oxygen, carbon dioxide, carbon monoxide, cyanide, hydroxyl amine, sodium azide, hydrogen sulfide, urethane, and mild oxidizing and reducing agents. None of these was found to produce any observable change in the absorption spectrum. The inertness of the chlorophyll-protein compound with respect to these reagents is in contrast to the well known behavior of such iron-porphyrin protein compounds as hemoglobin and catalase.

In contrast to the photolability of chlorophyll in organic solvents, the absorption spectrum of the pigment in the aqueous extracts was found to be stable to high light intensities for long periods. A solution kept at 20° C. was subjected to an intensity of about 200,000 meter candles for 1 hour without measurable effect on the absorption spectrum.

Effect of Organic Solvents.—As mentioned above, the leaf spectrum and chlorophyll dissolved in organic solvents show differences not only in the position but also in the relative intensities of the absorption bands. With an aqueous leaf extract at 20° C., the presence of low concentrations of acetone (10 per cent) does not produce any visible effect. At higher acetone concentrations (20-25 per cent), definite changes take place in the spectrum; the minor bands become more prominent and the main red band shifts slightly towards the blue. With 30 per cent acetone, the protein begins to precipitate and is complete at about 50 per cent acetone but with some color remaining in solution. At higher acetone concentrations the chlorophyll is rapidly extracted from the protein. The effect of the different acetone concentrations is influenced by the pH of the solution, higher concentrations being necessary to produce the same effect for alkaline solutions (pH 8.5 to 9) as compared with neutral ones. Higher temperatures increase the ease of chlorophyll extraction. Ethyl alcohol does not sensibly differ from acetone in its effect.

It is well known that ether will not extract chlorophyll from the leaf and that is equally true for the aqueous leaf extract. However, when the aqueous preparation is vigorously shaken with ether, the preparation is readily emulsified and the spectrum becomes that of free molecular chlorophyll in ether. The fluorescence is also very much brighter. Ether will extract chlorophyll quite readily from dried chloroplast preparations. The failure of ether to dissolve chlorophyll from the leaf or aqueous extracts can be explained by the low solubility of ether in water, just as with moderate acetone concentrations (35 per cent) the spectrum is changed but the chlorophyll not extracted.

Effect of Temperature.—Sorby discovered in 1872 that heating a leaf causes

a shift in the position of the main absorption band in the red. Willstätter and Stoll later showed that the spectrum of the boiled leaf is similar to that of chlorophyll in phytol or lecithin. Noack found that heating a leaf causes the fluorescence first to disappear and on more prolonged heating to reappear. He ascribed the disappearance of the fluorescence to the denaturation of the protein and its subsequent reappearance to the solution of the chlorophyll in some waxy component of the leaf. Mestre found the change in the leaf spectrum to be a function of both time and temperature very similar to those for ordinary protein denaturations.

Heating an aqueous extract of the leaf produces changes in spectrum and fluorescence identical with those directly observed on the leaf. A green protein coagulum is gradually formed on heating a neutral solution above 60° C., with the fluorescence becoming weaker. When the coagulum is evaporated to dryness, the spectrum is identical with that given by Willstätter and Stoll for chlorophyll in phytol; the fluorescence is much more intense than for an unheated control.

Aside from the fact that these heating experiments strongly indicate the linkage of chlorophyll to protein, they also provide excellent criteria for determining the native state of the pigment complex. As in experiments with the chlorophyll solvents, the changes which take place are clearly reflected in the character of the spectra and fluorescence.

Effect of Alkali.—At pH 9.0 the leaf extract is quite stable and shows no change in its solubility, precipitation properties, or spectrum. In M/10 NaOH, the band at 678 mμ slowly becomes weaker and a new band at 640 mμ appears; this corresponds to the saponification of the esterified groups which occurs in strongly alkaline solutions with molecular chlorophyll. At the same time, the band at 475 mμ shifts towards the shorter wave lengths, making more prominent the carotenoid band at 485–490 mμ. The rate of saponification seems to be a direct function of the hydroxyl ion concentration. In M/10 NaOH the effect can be detected only after some hours, while with 5 M alkali the reaction is complete in a few minutes. In M/10 alkali, the protein is gradually precipitated. With very strong alkali (5 M), a precipitate of denatured protein forms immediately. Protein denaturation and the change in spectrum appear to be roughly parallel.

Effect of Acid.—Addition of dilute acetic acid causes the complete precipitation of the protein at a pH between 4.5 and 5.0, with no apparent change in the spectrum. Further addition of acid to pH 2 gradually changes the green color to a yellowish green and finally a yellow to brown. The spectrum is that of phaeophytin; the main red band is much weaker, and strong bands appear at 540 and 610 mμ.

The protein precipitated at pH 4.5 is no longer resuspended by neutraliza-

tion; much larger quantities of alkali are required and the protein is suspended only at a pH above 9.0. The precipitation at 4.5 and resuspension at pH 9.0 may be repeated indefinitely and seems to be a useful method of separating the chloroplast material from the cytoplasmic proteins which do not precipitate (*cf.* Menke, 1938). However, the properties of the chloroplast protein aside from its spectrum are definitely changed by this procedure: (1) as already mentioned, it does not resuspend at pH 7.0 but only at much higher pH's; (2) it is readily precipitated by 10 per cent saturation with ammonium sulfate and cannot be resuspended; the original extract requires about 30 per cent saturation for precipitation; (3) boiling the extract for several minutes does not cause any precipitation unless it is brought to an acid pH; it may then be resuspended by adding alkali to bring the solution again to pH 9.0. A control sample at pH 7 not previously treated with acid will form a heavy coagulum by heating to boiling and will not resuspend regardless of the pH.

These changes brought about by treatment with dilute acid are those usually ascribed to protein denaturation. The chloroplast protein is much more sensitive to weakly acid solutions than most proteins.

Effect of Drying.—Leaf extracts were dried by suspending them in cellophane dialyzing tubing in front of an electric fan. In this way, a large volume of extract may be handled, and the extracts kept cool during the entire evaporation process. Small samples of extracts slowly dried *in vacuo* over sulfuric acid gave similar results.

The dried chloroplast material could not be redissolved or suspended in water or neutral buffer solutions, but could be partially suspended in borate buffer at pH 9.0 or with dilute alkali. In these alkaline solvents, the protein appeared to be modified in the same way as with the acid-precipitated material; *e.g.*, the protein could be precipitated by 10 per cent saturation with ammonium sulfate.

Both the chlorophylls and carotenoids are rapidly extracted from the dried material by acetone, alcohol, and ether. Petroleum ether and carbon disulfide extract most of the carotenoids readily and only little chlorophyll even after several hours.

Effect of Detergents.—Because of the insolubility of the chlorophyll-protein complex, the effect of adding various dispersing agents which clarify leaf extracts has been studied. Preparations of the chloroplast material in the detergent were prepared in two different ways. The most direct method was to add the detergent solution directly to the leaf extract. The other method was to add to the leaf extract about 5 per cent Filter-Cel and then filter through a thin layer of Filter-Cel on a Buchner funnel. The

yellow-brown filtrate is discarded, and the filter-cake then washed with water or neutral buffer solution until the filtrates show no yellow color. In this way, all of the water-soluble material extracted from the leaf may be removed. The filter-cake is then extracted with the detergent solution and filtered.

Extraction with a 1 to 5 per cent digitonin[2] solution after filtration yields a clear dark green solution which shows no trace of particles under an oil

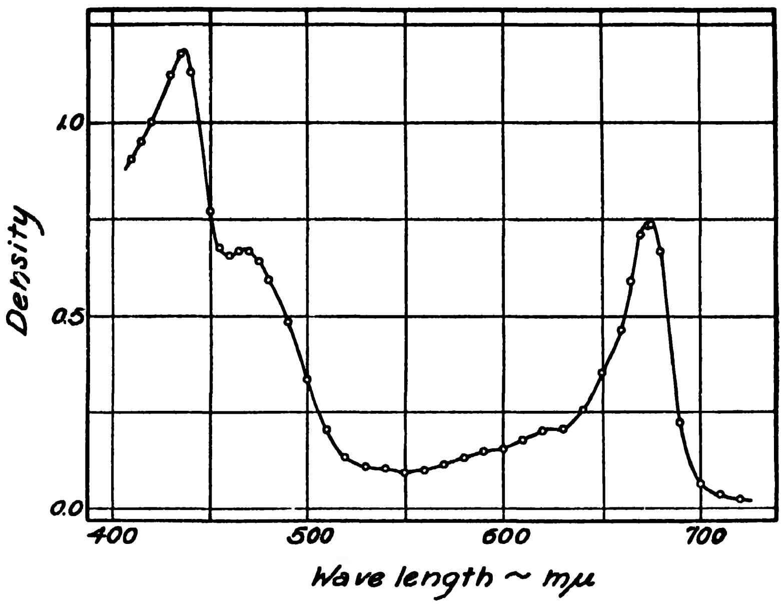

FIG. 2. The absorption spectrum of an extract from the spinach leaf prepared in 2 per cent digitonin and diluted 1 to 10 with distilled water. The data are given in Table II.

immersion lens. The absorption spectrum of a diluted solution is shown in Fig. 2 and the data are given in Table II. When this curve is compared with that of a direct aqueous leaf extract (Fig. 1), several differences are apparent. The absorption drops very rapidly on the long wave side of 700 mμ; it has already been pointed out that the apparent absorption in this region shown by an aqueous extract is due to light scattering. The removal of scattering is probably also responsible for the decrease in the

[2] The digitonin was obtained from Eimer and Amend, New York, as crystalline digitalin. This digitonin was dissolved by heating to a gentle boil when the solution becomes water clear. On cooling, the solution will remain clear for some weeks at room temperature. Over longer periods, some precipitation occurs.

relative height of the middle region of the spectrum. In addition, the pigment in digitonin shows a shift of the main red band from 677–678 mμ to 675 mμ, and of the minimum region of absorption from 560 mμ to 550 mμ. The sharper character of the band at 470 mμ in the digitonin solution is undoubtedly due to the removal of the yellowish impurities.

Solutions clarified by the addition of digitonin show a somewhat increased fluorescence when compared visually with a direct leaf extract. It is likely that the apparent increase in fluorescence may be due to the decrease in light scattering caused by the presence of the detergent.

TABLE II

Absorption Spectrum of Spinach Leaf Extract in Digitonin

Data of Fig. 2. Extract prepared in 2 per cent digitonin and diluted 1 to 10 with distilled water.

λ	Density	λ	Density	λ	Density
mμ		*mμ*		*mμ*	
720	0.0254	610	0.1794	480	0.5930
710	0.0346	600	0.1602	475	0.6414
700	0.0678	590	0.1506	470	0.6694
690	0.2218	580	0.1374	465	0.6670
680	0.6658	570	0.1182	460	0.6562
675	0.7370	560	0.0998	455	0.6734
673	0.7346	550	0.0974	450	0.7712
670	0.7086	540	0.1018	440	1.1294
665	0.5914	530	0.1090	435	1.1754
660	0.4654	520	0.1350	430	1.1202
650	0.3518	510	0.2054	420	1.0090
640	0.2590	500	0.3356	415	0.9522
630	0.2090	490	0.4854	410	0.9046
620	0.2034				

The precipitation properties of the chloroplast protein are distinctly modified by the presence of the digitonin. Even saturation with ammonium sulfate is quite ineffective. Most of the digitonin can be removed by ultrafiltration through a 3 per cent Bechold collodion membrane without loss of pigment, but prolonged dialysis is necessary for complete removal of the detergent. The absence of the digitonin in the dialysate can be readily tested by shaking vigorously since all of the detergents produce a persistent foaming. After dialysis, the pigment is readily precipitated by a tenth saturation with ammonium sulfate, and can be redissolved in digitonin solution. The pigment can also be precipitated by acidification to pH 4.5 and redissolved by buffer solution at pH 9.0. This process can be repeated indefinitely. In this respect, the properties of the pigment are similar to those produced by direct acid precipitation.

Bile salts (a purified mixture of sodium glycocholate and taurocholate) and sodium desoxycholate have also been used for dispersing the chloroplast pigment. The properties of the pigment in these detergents closely resemble those in digitonin solutions. For equivalent concentrations, the desoxycholate is somewhat more effective than either bile salts or digitonin. However, desoxycholate has the disadvantage of being insoluble at acid pH's and it tends to precipitate or gel even at slightly alkaline ones.

The absorption spectrum of the pigment in these detergents is almost identical with that found in digitonin. The only difference is that in both, the position of the main red band is shifted further towards the blue, and is found at 671 to 672 mμ.

Concentrated urea solutions (50 per cent) also clarify aqueous solutions of the chloroplast pigment. The absorption spectrum is identical with that of the pigment in digitonin with the main red absorption band at 675 mμ.

V

Relationship of Chlorophyll to Protein

If a true combination exists between chlorophyll and protein, there should be a definite quantitative relationship between them. This point has been investigated by purifying the chloroplast material in different ways, and then evaluating chlorophyll in relation to the dry weight, and in a few cases, to the chloroplast nitrogen as well.

Estimation of Chlorophyll Concentration.—The usual method of estimating chlorophyll colorimetrically by matching against a standard solution of chlorophyll is subject to the difficulty of obtaining chlorophyll solutions of known purity. Moreover, the absolute extinction coefficients of chlorophylls *a* and *b* are still subject to some revision although it does not appear likely that they will change very much. We have preferred to estimate chlorophyll by measuring the extinction at the maximum absorption at the red end of the spectrum where there is no interference by the yellow pigments of the leaf. Using the best absolute extinction values, it is then possible to compute the chlorophyll concentration.

Although the position of the absorption band in the aqueous preparations is different from that of chlorophyll in organic solvents, the same preparation has an identical extinction value in the aqueous extract clarified by digitonin, or in ether or petroleum ether.[3] This comparison was made by diluting an aliquot portion of the concentrated aqueous extract until the extract had several times the chlorophyll concentration suitable for spectrophotometric estimation. The extract was then diluted with a 5 per cent solution of digitonin until the final digitonin concentration was 1 or 2 per cent.

[3] In a preliminary communication (Smith, 1940) it was inadvertently stated that the "extinction value in water as protein compound, or in ether or petroleum ether" is the same. The statement should read "aqueous digitonin" in place of "water."

The chlorophyll from another sample of the extract was transferred to ether by adding ten volumes of acetone to precipitate the protein, washing with more acetone, and finally washing the chlorophyll into the ether by adding water. After several additional washings of the ether with water, the ether extract was brought to a definite volume and the chlorophyll estimated spectrophotometrically. The data of four separate experiments are given in Table III. Single determinations with ethanol and acetone as solvents are in agreement with the data for ether and petroleum ether.

The aqueous extract cannot be directly compared with the organic solvents since the former shows a large and variable loss of light caused by scattering, giving an appreciably higher extinction value. While digitonin has been used to eliminate this scattering, it is likely that other clarifying agents, such as bile salts, would also serve the same purpose. The absorption of the pigment in digitonin was found to follow the Lambert and Beer law over a tested concentration range of one to ten.

TABLE III

Comparison of Chlorophyll Absorption in Different Solvents

Experiment	Solvent	λ maximum	Density	Averages
		mμ		
1	Ether	660	1.14	
		661	1.16	
		660	1.17	1.16
	Digitonin	675	1.18	
		675	1.17	1.18
2	Petroleum ether	661	1.25	
		660	1.22	1.24
	Digitonin	675	1.25	1.25
3	Ether	660.5	1.67	
		660	1.81	
		660	1.80	1.76
	Digitonin	674.5	1.80	1.80
4	Ethanol	665	1.36	
	Acetone	663	1.32	
	Digitonin	675	1.40	

Accepting the findings of Willstätter and Stoll, it has been assumed that the leaf pigment contains chlorophylls *a* and *b* in a ratio of three to one. On this basis, values for the mixed pigments have been computed from the best available data. Using the molecular extinction coefficient ϵ where

$$\epsilon cd = \log_{10} I_0/I = D$$

the data of Zscheile (1934) give 5.4×10^4. When the data of Winterstein and Stein (1933) are converted from $\log_e$ to $\log_{10}$, the same value is obtained. MacKinney's (1940) recent data on chlorophylls *a* and *b* yield the value 5.6×10^4. Since the higher value indicates purer components, the absolute chlorophyll concentrations are calculated

in terms of this value. The most convenient method of expressing the relative values is as optical density (D) per mg. of dry weight per ml. of solution per cm. absorption. The percentage of chlorophyll is $D(900)(100)/5.6 \cdot 10^4$ or 1.61 D.

Purification.—Most of the preparations were purified by repeated salt precipitation and resuspension in slightly alkaline phosphate buffer. The spinach protein could be precipitated by 0.3 saturation with ammonium sulfate or by saturation with sodium chloride. *Aspidistra* protein could not be precipitated with sodium chloride but was precipitated by half saturation with ammonium sulfate. The ammonium sulfate was always added from a saturated solution made slightly alkaline (phenol red) with 0.1 N sodium hydroxide. It was necessary to carry out all of the manipulations in slightly alkaline solutions in the cold; otherwise denatured protein was obtained which could not be resuspended.

Determinations of the dry weight were made after the solutions were thoroughly dialyzed in cellophane tubing first against slightly alkaline phosphate buffer, and finally against distilled water. Dry weights were determined by evaporating an aliquot portion of the solution over a steam bath and finally by drying in an evacuated dry chamber over sulfuric acid.

Initial extracts of the leaves had a D/mg./ml./cm. between 1.2 and 1.4 both for spinach and *Aspidistra*. This is roughly one-fourth of the average value obtained for the purified material after three or four precipitations. There was generally only a small change in the D value after the second precipitation. The final values are given in Table IV.

In one experiment purification was effected in a different manner. The leaf extract was sedimented in an air-turbine concentration centrifuge at 8000 R.P.M. The preparation was sedimented twice, resuspending in M/10 Na_2HPO_4, and finally three times more, resuspending each time in distilled water. The precipitates were collected in a little distilled water. To a carefully measured volume, an equal volume of 5 per cent digitonin was added. This was centrifuged at 2500 R.P.M. for 15 minutes, and aliquot portions of the clear solution were taken for chlorophyll, dry weight, and nitrogen estimation. A sample of the digitonin solution was also taken for dry weight. Estimation showed that the digitonin preparation was nitrogen-free. The results of this experiment also given in Table IV are in keeping with the others.[4]

Table IV summarizes the purification data. Using the actual chlorophyll determinations, the total nitrogen values (micro Kjeldahl) were corrected for the 6.2 per cent nitrogen present in chlorophyll assessed on the

[4] Thanks are gratefully acknowledged to Dr. E. A. Kabat of Cornell University Medical School for the use of the air-turbine centrifuge and for his aid with this experiment.

basis of three parts of *a* to one of *b*. The remaining nitrogen was assumed to be protein nitrogen, using the customary factor 6.25. The average chlorophyll content of the isolated chloroplast material was 7.86 per cent. For the three experiments where nitrogen was determined, the protein content of the chloroplasts was 46.5 per cent in good agreement with the average value of 47.7 per cent found by Menke (1938) for spinach leaves.

The average chlorophyll content was 16.1 parts of chlorophyll per 100 parts of protein.[5] This is in contrast to the results of Granick (1938) and Mommaerts (1938). Granick found 27 parts of chlorophyll per 100 parts of protein calculated from his statement of 30 molecules of chlorophyll

TABLE IV

Relationship of Chlorophyll to Protein in Chloroplast

Species	Nitrogen	Protein N	Protein (protein N·6.25)	Density	Chlorophyll	Chlorophyll per 100 parts of protein	Method of purification
	per cent	*per cent*	*per cent*		*per cent*		
Spinacia	8.3	7.8	48.8	4.93	7.94	16.3	High speed centrifuging
				5.26	8.47		$(NH_4)_2SO_4$ precipitation
				4.88	7.86		" " "
				5.06	8.15		Sodium chloride precipitation
				5.40	8.69		" " "
Aspidistra	7.4	6.9	43.1	4.43	7.13	16.5	$(NH_4)_2SO_4$ precipitation
	8.1	7.6	47.5	4.56	7.34	15.5	" " "
				4.33	6.97		" " "
				5.07	8.16		" " "
				4.92	7.92		" " "
Averages		7.4	46.5	4.88	7.86	16.1	

per 100,000 molecular weight of protein. Mommaerts found about 5.5 parts of chlorophyll per 100 parts of protein. The decided discrepancy between the results of these two investigators and the data given here may be at least partly explained by the fact that Mommaerts removed the chlorophyll from the chloroplasts with ether and determined the dry weight of the ether-insoluble residue, assuming that it was entirely protein. Granick determined chlorophyll colorimetrically but did not specify his standard of comparison. If his standard was of lower purity than MacKinney's it would aid in explaining the difference.

[5] After this work had appeared in preliminary form, the paper of Menke (1940) became available in which he reported an average value of 17.2 parts of chlorophyll per 100 parts of protein. This is in excellent agreement with the value of 16.1 reported here when one considers the different methods used.

The value of 16.1 per cent chlorophyll may have to be lowered somewhat if the absolute extinction coefficients for pure chlorophylls *a* and *b* are found to be higher. This does not appear likely since the results of Zscheile, Winterstein and Stein, and MacKinney agree within 5 per cent. On the other hand, further purification of the chloroplast protein may necessitate some revision of this figure. Some of the chloroplast nitrogen may not belong to the chlorophyll protein. Our evidence is negative in that other purification methods were unsuccessful in changing the chlorophyll to dry weight ratio. The pigment was readily adsorbed at pH 6.6–6.8 by alumina cγ, and gelatinous calcium triphosphate but elution at pH 8 to 9.5 was unsuccessful. When partial adsorption was carried out by using an amount of adsorbent insufficient to remove all of the pigment, the remaining pigment did not differ from the starting material already purified by salt precipitations. Other adsorbents such as copper hydroxide and calcium hydroxide behaved similarly. At pH 6.6 the green pigment was not adsorbed by bone charcoal or kaolin, nor did these adsorbents remove enough impurities to change the chlorophyll to dry weight ratio.

A few attempts were made to obtain an independent estimate of the chlorophyll concentration by measuring the magnesium content of leaf extracts or purified material by the Titan yellow method after digestion with sulfuric acid or with nitric acid and H_2O_2. The values obtained, especially with the unpurified extracts, always gave chlorophyll estimations much higher than those found by the spectrophotometric method, indicating the presence of magnesium not bound in the chlorophyll molecule.

VI

DISCUSSION

From the evidence of the spectral and chemical properties of the chloroplast pigment, it seems certain the chlorophyll exists in the leaf as the prosthetic group of a definite protein. The constant proportionality of chlorophyll to protein must be regarded as one of the more important facts indicating this linkage in spite of the fact that some uncertainty remains attached to the absolute ratio.

It is still undetermined whether the large quantity of non-protein material associated with the chloroplast protein represents a molecular combination or only an association complex. If the entire complex is in molecular combination, then the average chlorophyll content of 7.86 per cent would indicate a minimum molecular weight of 11,500 for the complex. Using the chlorophyll-protein ratio of 16.1 to 100, the minimum molecular weight is

5600, or a little over three chlorophyll molecules for the Svedberg protein unit of 17,500. Because of the much smaller light absorption at the standard wave length of chlorophyll *b* compared to chlorophyll *a*, the three and a fraction may represent three molecules of chlorophyll *a* and one of *b*. In their analyses of the leaves of many green plants, Willstätter and Stoll found that the ratio of chlorophyll *a* to *b* seldom deviated from three to one. Using a different method of chlorophyll estimation, Winterstein and Stein found the same ratio. This suggests a definite combining ratio of three molecules of *a* and one of *b* in the same protein unit. Although many hypotheses have been advanced ascribing different functions to chlorophylls *a* and *b*, this is, we believe, the first suggestion to explain the constant ratio.

There is some doubt whether the carotenoids are also bound to protein. None of the purification methods which have been attempted has served to separate any of the chlorophyll or carotenoid components of the chloroplast. The fact that petroleum ether readily extracts the carotenoids but not chlorophyll from dried chloroplast material indicates that the carotenoids may be only loosely associated rather than bound by true chemical linkage. On the other hand, sedimentation studies in the ultracentrifuge (Smith and Pickels, unpublished) in the presence of sodium dodecyl sulfate reveal no separation of chlorophylls and carotenoids even though the protein is split into smaller units. The existence of carotenoid-protein compounds in nature such as the astacene compounds of Crustacea, and visual purple, shows that such combination is not unlikely.

Whether the close association of all the pigment components of the chloroplast is a loose one or is in the form of a giant molecule as postulated by Lubimenko, this association must be of importance in the photosynthetic mechanism. In any case, the combination of chlorophyll with protein must be taken into consideration in dealing with the problem of photosynthesis.

It is a real pleasure to acknowledge the generous help and many kindnesses of Professor D. Keilin while the author was a guest at the Molteno Institute, and to thank Professor Selig Hecht for his always available advice and criticism.

SUMMARY

1. Aqueous extracts of spinach and *Aspidistra* leaves yield highly opalescent preparations which are not in true solution. Such extracts differ markedly from colloidal chlorophyll in their spectrum and fluorescence. The differences between the green leaf pigment and chlorophyll in organic

solvents are shown to be due to combination of chlorophyll with protein in the leaf.

2. The effect of some agents on extracts of the chlorophyll-protein compound has been investigated. Both strong acid and alkali modify the absorption spectrum, acid converting the compound to the phaeophytin derivative and alkali saponifying the esterified groups of chlorophyll. Even weakly acid solutions (pH 4.5) denature the protein. Heating denatures the protein and modifies the absorption spectrum and fluorescence as earlier described for the intact leaf. The protein is denatured by drying. Low concentrations of alcohol or acetone precipitate and denature the protein; higher concentrations cause dissociation liberating the pigments.

3. Detergents such as digitonin, bile salts, and sodium desoxycholate clarify the leaf extracts but denature the protein changing the spectrum and other properties.

4. Inhibiting agents of photosynthesis are without effect on the absorption spectrum of the chlorophyll-protein compound.

5. The red absorption band of chlorophyll possesses the same extinction value in organic solvents such as ether or petroleum ether, and in aqueous leaf extracts clarified by digitonin although the band positions are different. Using previously determined values of the extinction coefficients of purified chlorophylls *a* and *b*, the chlorophyll content of the leaf extracts may be estimated spectrophotometrically.

6. It was found that the average chlorophyll content of the purified chloroplasts was 7.86 per cent. The protein content was 46.5 per cent yielding an average value of 16.1 parts per 100 parts of protein. This corresponds to a chlorophyll content of three molecules of chlorophyll *a* and one of chlorophyll *b* for the Svedberg unit of 17,500. It is suggested that this may represent a definite combining ratio of *a* and *b* in the protein molecule.

BIBLIOGRAPHY

Dhéré, C., La fluorescence en biochimie, Paris, Les Presses Universitaires de France, 1937.

French, C. S., The chromoproteins of photosynthetic purple bacteria, *Science*, 1938, **88**, 60.

French, C. S., The pigment-protein compound in photosynthetic bacteria. I. The extraction and properties of photosynthin, *J. Gen. Physiol.*, 1940, **23**, 469.

Granick, S., Chloroplast nitrogen of some higher plants, *Am. J. Bot.*, 1938, **25**, 561.

Hagenbach, E., Untersuchungen über die optischen Eigenschaften des Blattgrüns, *Ann. Phys. u. Chem.*, 1870, **121**, 245.

Hagenbach, E., Fernere Versuche über Fluorescenz, *Ann. Phys. u. Chem. Pogg.*, Jubelband, 1874, 303.

Herlitzka, A., Ueber den Zustand des Chlorophyll in der Pflanze und über kolloidales Chlorophyll, *Biochem. Z.*, Berlin, 1912, **38**, 321.

Hubert, B., The physical state of chlorophyll in the plastid, *Rec. trav. bot. néerl.*, 1935, **32**, 323.

Ivanovski, D., Ueber die Ursachen der Absorption-bänder im Blatt, *Ber. deutsch. bot. Ges.*, 1907, **25**, 416.

Ivanovski, D., Kolloidales Chlorophyll und die Verschiebung der Absorptionsbänder in lebenden Pflanzenblättern, *Biochem. Z.*, Berlin, 1913, **48**, 328.

Keilin, D., A comparative study of turacin and haematin and its bearing on cytochrome, *Proc. Roy. Soc. London, Series B*, 1926, **100**, 129.

Lubimenko, V., Les pigments des plastes et leur transformation dans les tissus vivants de la plante. 1. Les pigments des chloroplastes, *Rev. gén. bot.*, 1927, **39**, 547.

MacKinney, G., Criteria for purity of chlorophyll preparations, *J. Biol. Chem.*, 1940, **132**, 91.

Menke, W., Untersuchungen über das Protoplasma grüner Pflanzenzellen. I. Isolierung von Chloroplasten aus Spinatblättern, *Z. physiol. Chem.*, 1938, **257**, 43.

Menke, W., Untersuchungen über das Protoplasma grüner Pflanzenzellen. II. Der Chlorophyllgehalt der Chloroplasten aus Spinatblättern, *Z. physiol. Chem.*, 1940, **263**, 100.

Mestre, H., The investigation of the pigments of the living cell, in Contributions to marine biology, Stanford University Press, 1930, 170.

Meyer, K. P., Spektrometrische Untersuchungen über den Zustand des Chlorophylls in der Pflanze, in Extrakten und Reinpräparaten, *Helv. Phys. Acta*, 1939, **12**, 349.

Mommaerts, W. F. H. M., Some chemical properties of the plastid-granum, *Proc. k. Akad. Wetensch.*, 1938, **41**, 896.

Noack, K., Der Zustand des Chlorophylls in der lebenden Pflanze, *Biochem. Z.*, Berlin, 1927, **183**, 135.

Osborne, T. B., and Wakeman, A. J., The proteins of green leaves. I. Spinach leaves, *J. Biol. Chem.*, 1920, **42**, 1.

Osborne, T. B., The chemistry of the cell, *Leopoldina, Amerikaband*, 1928, 224.

Palladin V. I., Zur Physiologie der Lipoide, *Ber. deutsch. bot. Ges.*, 1910, **28**, 120.

Shlaer, S., A photoelectric transmission spectrophotometer for the measurement of photosensitive solutions, *J. Opt. Soc. America*, 1938, **28**, 18.

Smith, E. L., Solutions of chlorophyll-protein compounds (phyllochlorins) extracted from spinach, *Science*, 1938, **88**, 170.

Smith, E. L., Chlorophyll as the prosthetic group of a protein in the green leaf, *Science*, 1940, **91**, 199.

Sorby, H. C., On comparative vegetable chromatology, *Proc. Roy. Soc. London*, 1872, **21**, 442.

Stoll, A., Zusammenhänge zwischen der Chemie des Chlorophylls und seiner Funktion in der Photosynthese, *Naturwissenschaften*, 1936, **24**, 53.

Tschirch, A., Untersuchungen über das Chlorophyll, *Ber. deutsch. bot. Ges.*, 1883, **1**, 462.

Willstätter, R., and Stoll, A., Untersuchungen über Chlorophyll, Berlin, Julius Springer, 1913.

Winterstein, A., and Stein, G., Fraktionierung und Reindarstellung organischer Substanzen nach dem Prinzip der chromatographischen Absorptionsanalyse. II. Chlorophylle, *Z. physiol. Chem.*, 1933, **220**, 263.

Zscheile, F. P., Jr., An improved method for the purification of chlorophylls *a* and *b*; quantitative measurements of their absorption spectra; evidence for the existence of a third component of chlorophyll, *Bot. Gaz.*, 1934, **95**, 529.

THE ACTION OF SODIUM DODECYL SULFATE ON THE CHLOROPHYLL-PROTEIN COMPOUND OF THE SPINACH LEAF*

By EMIL L. SMITH**

(*From the Laboratory of Biophysics, Columbia University, New York, and the Molteno Institute, University of Cambridge, England*)

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I

INTRODUCTION

Evidence has been presented that in the green leaf chlorophyll is bound to protein by true chemical linkage (Smith, 1941). In order to elucidate additional properties of this compound, the effect of sodium dodecyl sulfate was studied. Sreenivasaya and Pirie (1938) demonstrated that the tobacco mosaic virus protein is split by sodium dodecyl sulfate into fragments of smaller size than the initial virus preparation, and at the same time, the nucleic acid is separated from the protein. Anson (1939) observed that various detergents including some which contain sodium dodecyl sulfate denature hemoglobin and egg albumin. Keilin and Hartree (1940) found that cytochrome *c* is reversibly changed by sodium dodecyl sulfate apparently affecting the linkage of the heme group to the protein since the absorption spectrum is modified.

II

Effect of Sodium Dodecyl Sulfate

When a solution of sodium dodecyl sulfate (SDS)[1] is added to an alkaline leaf extract, every trace of opalescence disappears and a brilliantly clear green solution is obtained. SDS thus has a similar action to digitonin or bile salts (Smith, 1941), but differs from these in its much greater effectiveness. The SDS clarified preparations show a clear red fluorescence which appears to be greater than that of the untreated leaf extract, but have a

* Part of this work has already been presented in a preliminary communication (Smith, 1940 *a*).

** John Simon Guggenheim Memorial Fellow (1938–1940).

[1] The SDS used in these experiments was part of a gift to Professor D. Keilin from Imperial Chemical Industries.

much smaller fluorescence than an equivalent concentration of free chlorophyll in acetone or ether.

The most striking action of the SDS is that in addition to clarifying the solution, magnesium is eliminated from the chlorophyll converting it to phaeophytin. The reaction is extremely rapid in weakly acid solutions

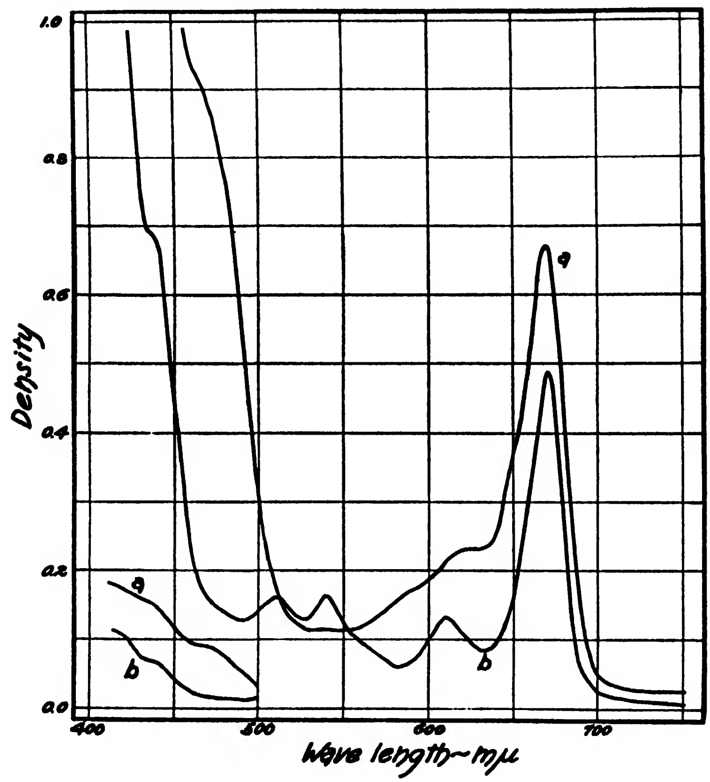

FIG. 1. The absorption spectra of the chloroplast pigment in SDS solutions at pH 8.90 (Curve *a*) and pH 5.30 (Curve *b*). In the short wave region of the spectrum, the curves have been plotted at a tenth of the measured density values. The data are given in Table I.

and takes place slowly in more alkaline solutions. This change is apparent by the striking color change from the original brilliant green first to an olive green and finally to a yellow or brown depending on the concentration of the pigment. In Fig. 1 are shown the absorption spectra of two solutions identical in all respects except that *a* was buffered at pH 8.9 and *b* at pH 5.3. The spectrum of the alkaline solution was measured immediately after addition of the SDS. No significant change occurred during the course of the measurements. After addition of the SDS solution to the acid buffered

solution (*b*), it was allowed to stand overnight to permit the reaction to go to completion.

The spectrum of the chlorophyll-protein compound even in the alkaline SDS solution shows striking changes. The maximum of the main red band which is at 678 in the leaf extract is shifted to 670 mμ. The prominent bands in the blue at 470 and 437 mμ are reduced in the SDS treated solution to slight inflections, as the entire curve appears to rise towards a maximum in the ultraviolet.

The spectrum of the acid SDS solution is plotted on the same density scale as the alkaline one. It shows a decided decrease in density at the red maximum which remains at 670 mμ, a pronounced shift of the entire blue absorption region towards the ultraviolet, and the appearance of three new absorption bands at 610, 540, and 510 mμ. The absorption minima are at 632.5, 583, 527.5, and 490 mμ. The actual measurements of both spectra are given in Table I. Comparison of the acid spectrum with the absorption spectrum of phaeophytin *a* in dioxane (Stern and Wenderlein, 1936) shows that phaeophytin, the magnesium-free derivative of chlorophyll, has been formed. In dioxane, the band maxima are from 1 to 5 mμ further towards the blue end of the spectrum than for the pigment in aqueous solution.

Ultrafiltration of a clarified extract through a 3 per cent Bechold collodion membrane or dialysis does not result in any loss of chlorophyll or carotenoid pigment showing that these pigments remain attached to large molecules. After prolonged dialysis against alkaline buffer solutions, the SDS may be nearly completely removed. The solution remains clear. The pigment and protein are precipitated by acidifying with dilute acetic acid, and can be redissolved with alkali. The complex is precipitated from solution by a tenth saturation with ammonium sulfate. This precipitate cannot be redissolved in water or neutral buffer, but is readily dissolved in SDS solution. The low concentration of salt required and its subsequent insolubility indicate that the protein is denatured by SDS. This is true for the protein compound whether the prosthetic group is phaeophytin or chlorophyll. No separation of the pigment from the protein can be obtained by fractional precipitation.

SDS readily dissolves the protein denatured by boiling. However, in these preparations, phaeophytin is formed much more rapidly than in control preparations buffered at the same pH. In one experiment, two 3 ml. samples of a leaf extract were strongly buffered at pH 8. One was boiled for 5 minutes, cooled to room temperature, and 1 ml. of 5 per cent SDS added to both solutions. In the boiled solution, phaeophytin formation

was complete in less than 3 hours, while the control solution still showed some green color and incomplete phaeophytin formation after 20 hours.

TABLE I

The Effect of Sodium Dodecyl Sulfate on the Absorption Spectrum of the Chloroplast Pigment. Data of Fig. 1

Measurements were made with the spectrophotometer of Shlaer (1938).

λ	Density at pH 8.90	Density at pH 5.30	λ	Density at pH 8.90	Density at pH 5.30
mμ			*mμ*		
750	0.0244	0.0052	560	0.1168	0.0964
740	0.0260	0.0088	550	0.1127	0.1220
730	0.0244	0.0096	542	—	0.1612
720	0.0260	0.0116	541	—	0.1628
710	0.0319	0.0152	540	0.1151	0.1628
700	0.0462	0.0216	538	—	0.1600
690	0.1327	0.0576	530	0.1134	0.1304
685	0.2369	0.1156	527.5	—	0.1284
680	0.4116	0.2508	525	—	0.1288
675	0.5855	0.4172	520	0.1268	0.1372
672	0.6426	0.4744	511	—	0.1612
671	—	0.4848	510	0.1772	0.1616
670	0.6712	0.4872	509	—	0.1612
669	0.6712	0.4848	500	0.3066	0.1436
668	0.6695	—	495	—	0.1316
665	0.6375	0.4232	490	0.5359	0.1276
660	0.5158	0.3312	485	—	0.1320
650	0.3721	0.1636	480	0.7619	0.1404
640	0.2545	0.0944	475	—	0.1480
635	—	0.0840	470	0.8854	0.1592
632.5	—	0.0824	465	—	0.1808
630	0.2302	0.0840	460	0.9408	0.2324
625	0.2302	—	455	—	0.3188
620	0.2302	0.1108	450	1.1567	0.4304
612	—	0.1308	445	—	0.5684
610	0.2134	0.1320	440	1.4860	0.6768
608	—	0.1304	435	—	0.6904
600	0.1873	0.0984	430	1.5750	0.7612
590	0.1722	0.0664	425	—	0.9280
585	—	0.0616	420	1.7302	1.0836
*583	—	0.0604	415	—	1.1252
580	0.1537	0.0624	410	1.8287	—
570	0.1336	0.0784			

This suggests that the action of the SDS takes place at several different linkages in the chlorophyll-protein compound. Anson has shown that Duponol WA, which consists mostly of SDS, readily denatures hemoglobin with liberation of sulfhydryl groups. Similarly it appears that SDS de-

natures the chlorophyll-protein compound splitting linkages which facilitates the removal of magnesium from the molecule. When denaturation is first accomplished by heating, the magnesium is rapidly removed.

It should be emphasized that SDS removes magnesium from the chlorophyll-protein compound in neutral and in slightly alkaline solutions. Heretofore, acid has been used to remove magnesium from chlorophyll dissolved in organic solvents. SDS is without effect on chlorophyll dissolved in acetone. Magnesium is removed by SDS from colloidal chlorophyll suspended in neutral solutions but at a slower rate than from the chlorophyll-protein compound.

III

Kinetics

1. Methods and Procedure.—All of the measurements were made with unpurified extracts of spinach leaves prepared as already described (Smith, 1941). The conversion of the pigment from chlorophyll to phaeophytin is readily observed spectrophotometrically because of the pronounced differences in the absorption spectra of the two pigments. The changes were followed at three separate wave lengths: at 670 mμ, the principal maximum in the red where a large decrease in density takes place (Fig. 1), similarly at 610 mμ, the maximum of a phaeophytin absorption band; and at 540 mμ where an increase in density takes place and a new absorption band appears. The measurements were made using the sensitive photoelectric spectrophotometer of Shlaer (1938).

After several preliminary experiments, all of the experiments were carried out over a period of 6 days on a single extraction which was kept in the refrigerator; no sensible change occurred in the extract during this period. Measurements were made at five pH values and at six different concentrations of sodium dodecyl sulfate, making ten runs in all since one run served for both series. The experiments of both series were made in random order.

The procedure was as follows: A sample of the extract was removed from the refrigerator and allowed to come to room temperature. To 0.5 ml. of extract were added 1.0 ml. of the appropriate sodium phosphate buffer (in one experiment sodium acetate buffer was used), a quantity of water where it was necessary to bring the solution to the final volume of 3 ml., and finally the sodium dodecyl sulfate solution. The final buffer concentrations were always tenth molar. The solution was mixed rapidly and pipetted into an absorption cell of 5 mm. optical thickness. Measurements were then carried out as rapidly as possible at the three wave lengths. In all cases, the pH of a sample of the reaction mixture was measured with the glass electrode. The measurements were made at room temperature (22–24°C.).

2. Effect of pH.—The change in density at 670 mμ at different pH's is shown in Fig. 2 and the data are given in Table II. For the data of pH 7.96, the correct time values are twice those in the figure. It is apparent that there is a large change in the rate of the reaction depending on the pH of the solution. At pH 7.96 nearly 5 hours (294 min.) are required for half

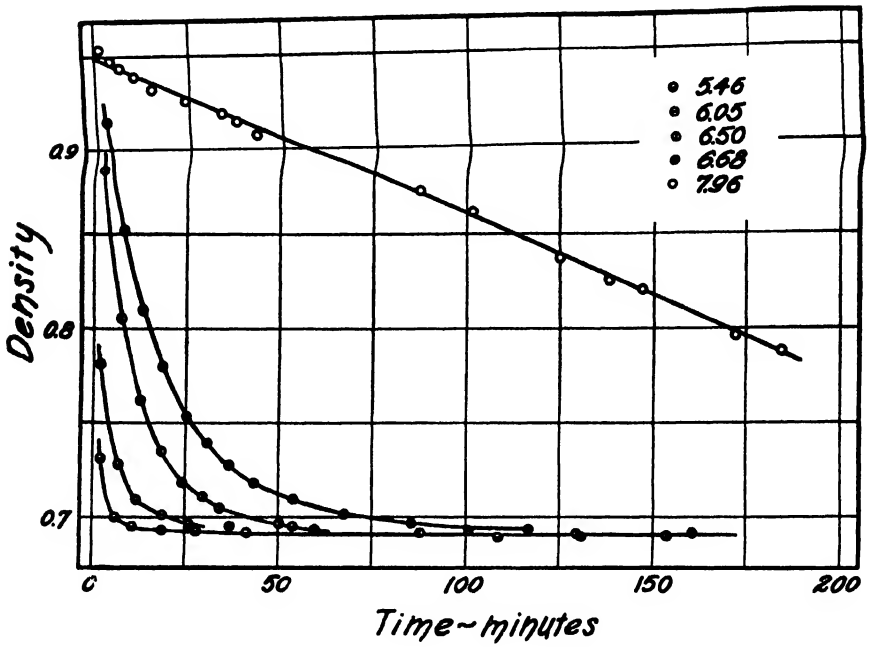

FIG. 2. Density changes at 670 mμ in solutions of different pH. For the data of pH 7.96 the plotted time values should be doubled. The numerical values are in Table II.

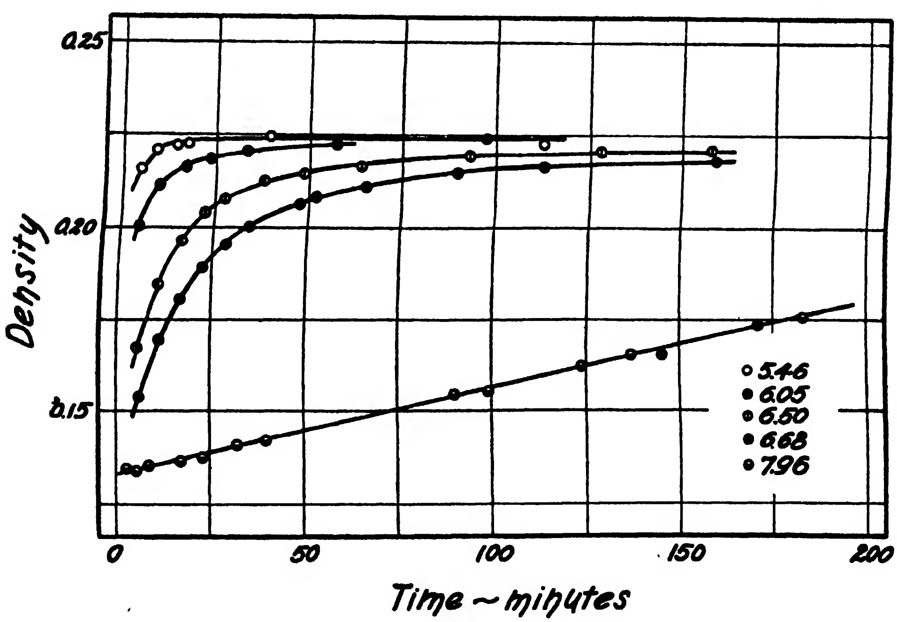

FIG. 3. Density changes at 540 mμ in solutions of different pH. For the data of pH 7.96 double the plotted time values. The data are given in Table III.

TABLE II

Change in Density at 670 mμ at Different pH's

Data of Fig. 2. SDS concentration constant at 1.67 per cent. Solution at pH 5.46 in 0.1 M sodium acetate buffers; others in 0.1 M sodium phosphate buffers.

pH = 5.46		pH = 6.05		pH = 6.50		pH = 6.68		pH = 7.96	
Time	Density	Time	Density	Time	Density	Time	Density	Time	Density
min.		*min.*		*min.*		*min.*		*min.*	
2.2	0.7308	1.7	0.7812	2.2	0.8860	2.5	0.9144	2.0	0.9536
6.3	0.7000	7.0	0.7280	7.5	0.8056	8.0	0.8520	7.5	0.9472
10.8	0.6952	11.8	0.7088	12.7	0.7624	13.3	0.8096	13.0	0.9436
18.9	0.6932	19.0	0.7008	18.5	0.7344	18.7	0.7800	20.0	0.9392
28.0	0.6928	26.0	0.6960	24.3	0.7184	25.2	0.7536	29.7	0.9320
41.5	0.6916	37.0	0.6948	29.7	0.7108	30.8	0.7392	47.5	0.9248
109.	0.6888	54.0	0.6948	34.3	0.7044	36.7	0.7276	67.0	0.9184
		101.	0.6924	50.2	0.6964	43.7	0.7180	75.0	0.9140
		131.	0.6892	60.0	0.6936	54.0	0.7088	85.5	0.9072
				88.0	0.6912	67.7	0.7016	175.	0.8728
				130.	0.6908	85.7	0.6964	203.	0.8616
				154.	0.6900	117.	0.6932	250.	0.8372
						161.	0.6916	276.	0.8248
						255.	0.6900	294.	0.8200
								344.	0.7960
								369.	0.7880

TABLE III

Change in Density at 540 mμ at Different pH's

Data of Fig. 3. Measurements made alternately with those given in Table II using the same solutions.

pH = 5.46		pH = 6.05		pH = 6.50		pH = 6.68		pH = 7.96	
Time	Density	Time	Density	Time	Density	Time	Density	Time	Density
min.		*min.*		*min.*		*min.*		*min.*	
5.0	0.2160	5.0	0.2008	5.7	0.1672	6.2	0.1542	5.5	0.1344
9.0	0.2208	10.0	0.2116	10.8	0.1848	11.5	0.1694	10.9	0.1340
14.3	0.2220	17.0	0.2168	16.6	0.1964	16.8	0.1802	17.3	0.1352
17.2	0.2224	23.7	0.2184	22.5	0.2040	22.7	0.1894	34.7	0.1364
39.5	0.2244	33.3	0.2204	27.7	0.2076	28.8	0.1954	46.0	0.1376
113.	0.2224	57.5	0.2224	38.0	0.2124	34.5	0.2002	64.5	0.1408
		97.5	0.2240	48.5	0.2144	47.7	0.2062	80.0	0.1420
				64.0	0.2168	52.0	0.2082	180.	0.1544
				93.0	0.2192	65.5	0.2110	198.	0.1556
				128.	0.2208	89.7	0.2150	248.	0.1624
				158.	0.2212	113.	0.2170	274.	0.1656
						159.	0.2182	291.	0.1656
						251.	0.2210	342.	0.1736
								366.	0.1756

of the total density change. On the other hand, at pH 5.46 the reaction is practically complete in 10 minutes. It has not been feasible to measure the rate in more acid solutions because of the time required for mixing and transferring the solutions to the spectrophotometer.

In Fig. 3 and Table III are presented the data which were obtained on the same solutions at 540 mμ. The character of the data is exactly the same as those at 670 mμ except that they show an increase in density instead of a decrease. The precision of the measurements with the spectrophotometer used is well illustrated by these data since the density changes at this

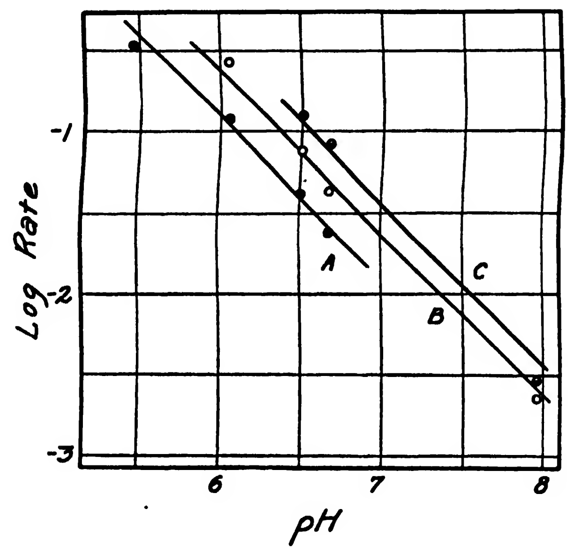

FIG. 4. Rate of phaeophytin formation as a function of pH at three density values: *A*, 0.72; *B*, 0.76; *C*, 0.80. These are the data for 670 mμ and are given in Table IV.

wave length are very small. The data obtained at 610 mμ have been omitted since they are so similar in character to those at 670 mμ. It is evident that the pH of the solution has little or no effect on the final phaeophytin spectrum since the final density is the same at all pH's both at 670 and 540 mμ; this is likewise true for the data at 610 mμ.

The influence of pH on the rate of the reaction can be estimated from the time required to reach a given density value at any one pH. In Fig. 4 are plotted the log rates (-log times) *versus* the pH, for three different density values. The lines drawn through the data have a slope of minus one indicating that the log rate is inversely proportional to the pH, or that the rate of the reaction is directly proportional to the hydrogen ion concentration. In other words, in the presence of sodium dodecyl sulfate the removal

of magnesium of the chloroplast pigment appears to be influenced by a simple hydrogen ion catalysis. The data in Fig. 4 are given in Table IV together with the data obtained from the measurements at 540 mμ. The effect of hydrogen ion concentration is identical at the three measured wave lengths.

3. Effect of Sodium Dodecyl Sulfate Concentration.—In Fig. 5 and Table V are presented the data obtained for the change in density at 670 mμ. The

TABLE IV

Rate of Phaeophytin Formation As a Function of pH

The data of 670 mμ are shown graphically in Fig. 4. These values were obtained by interpolation from the measurements given in Tables II and III. The value in brackets was obtained by extrapolation.

Wave length	pH	Density = 0.7200		Density = 0.7600		Density = 0.800	
		Time	−log time	Time	−log time	Time	−log time
mμ		*min.*		*min.*		*min.*	
670	5.46	2.7	−0.431	—	—	—	—
	6.05	8.4	−0.924	3.7	−0.568	—	—
	6.50	24.0	−1.380	13.1	−1.117	8.0	−0.903
	6.68	41.4	−1.617	23.3	−1.367	15.0	−1.176
	7.96	—	—	[440.]	[−2.644]	340.	−2.532
		Density = 0.2200		Density = 0.1700			
		Time	−log time	Time	−log time		
		min.		*min.*			
540	5.46	8.2	−0.914	—	—		
	6.05	30.8	−1.489	—	—		
	6.50	105.	−2.021	6.3	−0.799		
	6.68	217.	−2.337	11.7	−1.068		
	7.96	—	—	314.	−2.497		

plotted time values should be doubled for the data at the lowest SDS concentration (0.0209 per cent). The solutions were all buffered at pH 6.50. The curves show a marked effect of SDS concentration when the concentration is low. With these solutions there was no noticeable difference in character or rate of clarification of the solutions. When SDS was added to make the final concentration 0.01 per cent, or half that of the lowest concentration given in Fig. 5, no clarification of the solution was obtained. It was not possible to measure this solution spectrophotometrically. There seems to be a fairly abrupt transition below the least effective concentration of SDS indicating a threshold effect.

Relative rates of phaeophytin formation as a function of SDS concentra-

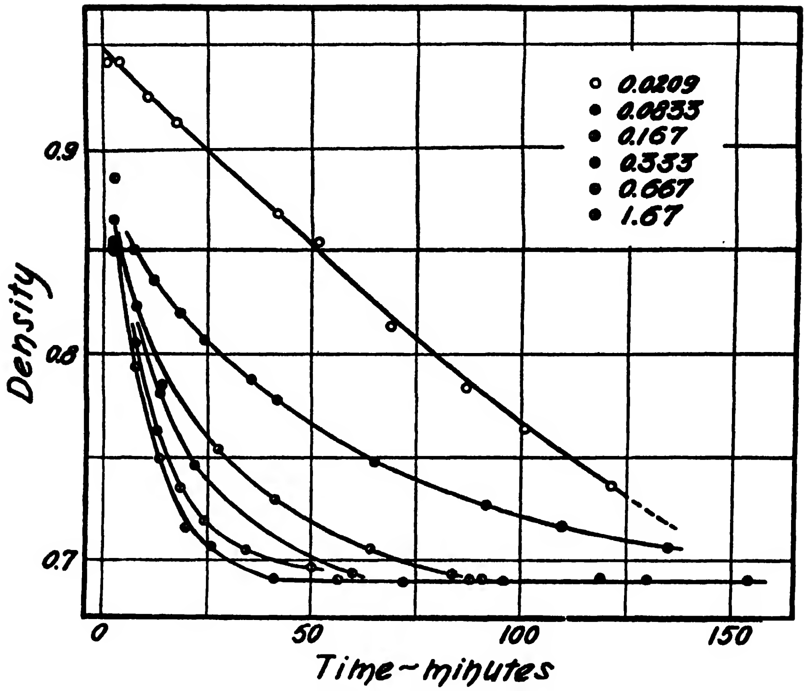

FIG. 5. Density changes at 670 mμ at different SDS concentrations, and at a constant pH of 6.50. For the data of 0.0209 per cent SDS, the correct time values are twice those plotted. The data are given in Table V.

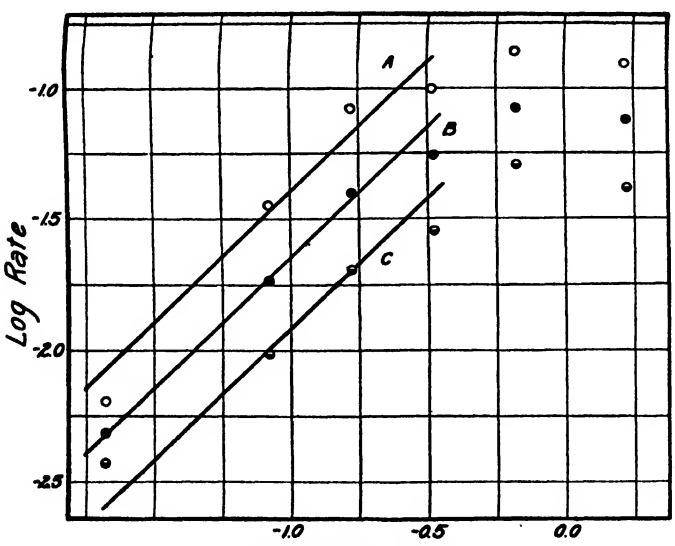

FIG. 6. Rate of phaeophytin formation as a function of SDS concentration at three different density values: *A*, 0.80; *B*, 0.76; *C*, 0.72. The data are given in Table VI.

tion were obtained from the data shown in Fig. 5 by the same procedure used for the pH data. The data obtained are given in Table VI. In Fig. 6 these data are plotted as log rate *versus* log SDS concentration. The lines

TABLE V

Change in Density at 670 mμ at Different Concentrations of Sodium Dodecyl Sulfate

Data of Fig. 5. pH was constant at 6.50 in 0.1 M sodium phosphate buffer. SDS concentrations are given in per cent.

[SDS] = 0.0209		[SDS] = 0.0833		[SDS] = 0.167		[SDS] = 0.333		[SDS] = 0.667		[SDS] = 1.67	
Time	Density	Time	Density	Time	Density	Time	Density	Time	Density	Time	Density
min.		*min.*		*min.*		*min.*		*min.*		*min.*	
1.8	0.9418	2.0	0.8540	2.1	0.8500	2.3	0.8642	2.0	0.8529	2.2	0.8860
7.0	0.9413	7.0	0.8496	7.9	0.8224	8.0	0.8222	7.5	0.7936	7.5	0.8056
21.0	0.9250	12.0	0.8348	14.0	0.7844	13.5	0.7802	13.3	0.7488	12.7	0.7624
34.7	0.9127	18.3	0.8188	27.7	0.7540	22.0	0.7460	20.0	0.7157	18.5	0.7344
83.7	0.8679	24.3	0.8064	41.1	0.7292	72.0	0.6889	26.0	0.7068	24.3	0.7184
103.	0.8534	35.8	0.7868	64.3	0.7056	96.0	0.6900	41.3	0.6911	29.7	0.7108
138.	0.8130	41.8	0.7772	83.7	0.6936	119.	0.6917	56.8	0.6900	34.3	0.7044
174.	0.7834	65.3	0.7480					91.0	0.6905	50.2	0.6964
202.	0.7638	91.8	0.7264							60.0	0.6936
243.	0.7363	110.	0.7164							88.0	0.6912
		135.	0.7064							130.	0.6908
										154.	0.6900

TABLE VI

Influence of Sodium Dodecyl Sulfate Concentration on Rate of Phaeophytin Formation

Data of Fig. 6. These values were obtained by interpolation from the data of Table V. The value in brackets is an extrapolated one.

Sodium dodecyl sulfate concentration	Log SDS concentration	Density = 0.7200		Density = 0.7600		Density = 0.8000	
		Time	−log time	Time	−log time	Time	−log time
per cent		*min.*		*min.*		*min.*	
0.0209	−1.780	[268.]	[−2.428]	208.	−2.318	156.	−2.193
0.0833	−1.079	103.	−2.013	54.5	−1.736	28.	−1.447
0.167	−0.777	49.	−1.690	25.	−1.398	12.	−1.079
0.333	−0.478	35.	−1.544	18.	−1.255	10.	−1.000
0.667	−0.176	19.5	−1.290	11.9	−1.076	7.2	−0.857
1.67	0.223	25.	−1.380	13.1	−1.117	8.0	−0.903

drawn have a slope of unity, and show that the rate is directly proportional to the SDS concentration within the precision of the data. The levelling of the data at the higher SDS values indicates that under the conditions of these experiments 0.4 per cent SDS produces the maximum rate.

4. *Effect of Temperature.*—The effect of temperature was observed only in a qualitative way. Aliquot portions of the same reaction mixture were

taken, exposed to different temperatures, and the color changes of the solutions observed visually. Temperature has an extremely large effect on the rate of the reaction. For the region between 20 and 30°C., the Q_{10} may be as high as 4 or 5. The high temperature coefficient may be only the usual high Q_{10} for protein denaturation since prior denaturation by boiling increases the rate of SDS action as described in Part II of this paper.

IV

DISCUSSION

The experiments of ultrafiltration, dialysis, and fractional precipitation show that the prosthetic group remains attached to the protein regardless of the presence or absence of magnesium in the molecule. This has been confirmed by an ultracentrifugal study of the solutions which showed in addition that the protein is split into particles of low molecular weight (Smith, 1940 *b*; Smith and Pickels, 1941). The action of SDS on the chlorophyll-protein compound differs from its action on the virus of tobacco mosaic disease; in the latter case, Sreenivasaya and Pirie showed not only a splitting of the protein, but also a separation of the prosthetic group (nucleic acid) from the protein.

The effect of SDS on cytochrome *c* (Keilin and Hartree) shows some similarity to the present experiments. Here also the prosthetic group remains attached to the protein, and it is the linkage of the metal, iron, which is apparently modified. However, with cytochrome *c* the effect was found to be reversible on removal of the SDS, while with the chlorophyll-protein compound, no reversal could be obtained.

The effect of pH shows that the lability of the magnesium atom is increased by the splitting and denaturing action of the SDS, and that it may not be the SDS itself which causes the removal of the metal. This is similar to the effect found by Inman and Crowell (1939) who observed that when trypsin is allowed to act on a leaf extract, the formation of phaeophytin by acid is enhanced. Nevertheless, when the chlorophyll-protein compound is split by digitonin or bile salts, no phaeophytin formation takes place even at pH 4.5. It appears that the SDS attacks different linkages in the molecule than those affected by digitonin or bile salts.

Since phaeophytin remains attached to the protein, it seems as though magnesium can play little part in binding the prosthetic group to the smaller protein units. On the other hand, the change in the spectrum produced by SDS at pH 9.0 indicates some modification, unless this effect can be ascribed wholly or in part to the solvent action of the SDS. The chlorophyll groups are probably oriented to the hydrophobic part of the SDS molecule

dissolving the chlorophyll in what may be considered an organic solvent. This may explain the shift of the red band from 678 mμ to 670 mμ, and also the modification of the absorption spectrum in the blue where the carotenoids as well as the chlorophyll would tend to disperse in the paraffin groups of the SDS.

It is too early to speculate much concerning the exact linkage of the chlorophyll to the protein. Linkages appear to be possible through the formyl group of chlorophyll *b*, the vinyl group, the labile hydrogen atoms of Stoll, and the magnesium. From the SDS action, it seems likely that the magnesium plays no rôle in binding chlorophyll to the smaller protein fragments, although it may be concerned in binding the intact molecule. This is indicated by the extreme lability of the magnesium in the presence of SDS and its stability in the presence of other detergents which also split the protein.

SUMMARY

1. Sodium dodecyl sulfate (SDS) attacks the chlorophyll-protein compound modifying its protein properties and absorption spectrum.

2. In the presence of SDS, chlorophyll is quantitatively converted to phaeophytin; *i.e.*, magnesium is removed from the molecule. This reaction, measured spectrophotometrically, proceeds at a rate directly proportional to the hydrogen ion concentration. At constant pH, the rate is proportional to the SDS concentration until a maximum rate is achieved.

3. The chlorophyll or phaeophytin (depending on the pH) remains attached to the protein, since the prosthetic group cannot be separated by ultrafiltration, dialysis, or fractional precipitation.

4. This suggests that the magnesium plays no part in binding chlorophyll to the split protein fragments, but may be concerned in binding the larger units, since the metal becomes extremely labile when the protein is split.

BIBLIOGRAPHY

Anson, M. L., The denaturation of proteins by synthetic detergents and bile salts, *J. Gen. Physiol.*, 1939, **23**, 239.

Inman, O. L., and Crowell, M. L., Condition of chlorophyll in the leaf, *Plant Physiol.*, 1939, **14**, 388.

Keilin, D., and Hartree, E. F., Properties of cytochrome *c*, *Nature*, 1940, **145**, 934.

Shlaer, S., A photoelectric transmission spectrophotometer for the measurement of photosensitive solutions, *J. Opt. Soc. America*, 1938, **23**, 18.

Smith, E. L., Chlorophyll as the prosthetic group of a protein in the green leaf, *Science*, 1940 *a*, **91**, 199.

Smith, E. L., An ultracentrifugal study of the action of some detergents on the chlorophyll-protein compound of spinach, *Am. J. Physiol.*, 1940 *b*, **129**, 466.

Smith, E. L., The chlorophyll-protein compound of the green leaf, *J. Gen. Physiol.*, 1941, **24**, 565.

Smith, E. L., and Pickels, E. G., The effect of detergents on the chlorophyll-protein compound of spinach as studied in the ultracentrifuge, unpublished.

Sreenivasaya, M., and Pirie, M. W., The disintegration of tobacco mosaic virus preparations with sodium dodecyl sulphate, *Biochem. J.*, London, 1938, **32**, 1707.

Stern, A., and Wenderlein, H., Über die Lichtabsorption der Porphyrine. IV, *Z. phys. Chem.*, 1936, *Abt. A*, **175**, 405.

STUDIES ON CELL METABOLISM AND CELL DIVISION

V. Cytochrome Oxidase Activity in the Eggs of Arbacia punctulata

By M. E. KRAHL, A. K. KELTCH, C. E. NEUBECK, and G. H. A. CLOWES

(*From the Lilly Research Laboratories, Indianapolis, Indiana, and the Marine Biological Laboratory, Woods Hole, Massachusetts*)

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In previous papers of this series (1–4) and allied publications, a study has been made of the effects of various agents on the respiration and cell division of the fertilized eggs of the sea urchin *Arbacia punctulata*. These experiments were part of a program directed toward gaining some insight into the respective rôles of individual enzyme systems in the utilization of oxidative energy for developmental processes in the sea urchin egg and other cells. For such investigations fertilized sea urchin eggs are especially suitable, owing to the fact that they depend almost exclusively on oxygen uptake for consumption of foodstuff, having little or no aerobic glycolysis or other metabolic activity of the anaerobic type under the conditions of experiment employed, even in the presence of cyanide and other respiratory inhibitors.

It was shown (3) that inhibition of division of fertilized *Arbacia* eggs took place at a level of respiratory inhibition which varied according to the type of inhibitor used. The significance of these experiments could not be assessed in terms of the individual oxidative enzymes of the eggs because, though effects of respiratory inhibitors on certain enzyme systems in other plant and animal cells have been worked out in detail in a few instances, no single known oxidative enzyme has been positively identified as being present in fertilized *Arbacia* eggs. For example, although it has been assumed, from the fact that the respiration of fertilized *Arbacia* eggs is partially poisoned by cyanide or carbon monoxide and stimulated by *p*-phenylenediamine, that such eggs contain a cytochrome oxidase-cytochrome system comparable to that in many other cells, no cytochrome bands have been observed in the eggs (4–6), and the presence of an enzyme system capable of oxidizing reduced cytochrome has not heretofore been demonstrated with certainty, though Ball and Meyerhof (5) recorded indications of the presence of such an enzyme in unfertilized *Arbacia* eggs.

The present paper is one of a series designed to correct this deficiency in

knowledge of respiratory enzymes in *Arbacia* eggs. It reports experiments to show that the eggs contain an enzyme which can oxidize reduced cytochrome c. This enzyme occurs in nearly equal amounts in unfertilized and fertilized *Arbacia* eggs, being present in both in a concentration sufficient to account for the respiration of the fertilized eggs even under the maximum degree of respiratory stimulation yet observed (1). The enzyme is inhibited—though in some instances to a degree differing greatly from that of the egg respiration—by cyanide, carbon monoxide, azide, and hydrogen sulfide, but not by such copper inhibitors as sodium diethyldithiocarbamate or 8-hydroxyquinoline.

In extension of previous experiments (4, 5, 7), a further effort to demonstrate the presence of cytochrome c and succinic dehydrogenase has shown that these oxidative catalysts, if present at all, occur in fertilized *Arbacia* eggs in concentrations disproportionately small in relation to the cytochrome oxidase activity, and too small to be of any apparent significance for the respiration of the egg.

From these experiments, certain tentative suggestions can be made regarding the probable nature of the oxidative systems operative in the fertilized *Arbacia* eggs.

EXPERIMENTAL METHODS

The cytochrome oxidase was prepared and used essentially according to Stotz (8) with the substitution of glycylglycine for part of the phosphate buffer in the test system and the use of a pH of 6.8 $\pm$ 0.1 to make the pH of the extraction and test system conform to what is believed from experiment to be the pH of the aqueous phase of the egg cytoplasm.

The details of the oxidase preparation were as follows: Ripe, mature eggs were obtained at Woods Hole during July and August, 1940 and, where necessary, fertilized according to methods reported in previous papers of this series. The volume of the eggs was, in every case, determined on the unfertilized eggs by the hematocrit method (9) (2700 times gravity for 5 minutes). Each cubic centimeter of eggs corresponded to approximately 5 $\times$ 10^6 eggs. The cytochrome oxidase values per cubic centimeter of eggs were converted to a wet weight and a dry weight basis using an egg density of 1.08 (10) and an egg solid content of 18 per cent (11). To carry out the preparation, the eggs, either unfertilized or at 30 minutes after fertilization at 20°C., were packed tightly by centrifuging at 2000 times gravity for 10 minutes. The eggs were then cytolyzed with 0.067 M Na_2HPO_4, using 5 cc. of phosphate solution for each gram of eggs; the resulting suspension was ground in a mortar with acid-washed sea sand at 5°C. for 20–25 minutes, using 0.4 gm. sand for each gram of eggs. The brei was then decanted from the sand, placed in cellophane tubing, and dialyzed overnight (22 hours) at 8°C. against 0.1 M phosphate buffer (pH 6.9) to reduce the concentration of any unidentified oxidizable substrates which might give large blank values in the manometric experiments. The resulting brei, after dilution with 0.1 M phosphate to a volume of 6.7 cc. per gm. of eggs,

was used as the enzyme preparation in the cytochrome oxidase experiments. It is of parenthetical interest that the liquid outside the dialysis tubes was pale yellow, and not red, at the end of the dialysis period, indicating that the echinochrome, though freely soluble in water, was tightly bound by the residual proteins of the brei.

Cytochrome c was prepared in this laboratory from beef hearts, according to the method of Keilin and Hartree (12), by Mr. T. V. Parke. After reduction with sodium hydrosulfite ($Na_2S_2O_4$) the cytochrome c was standardized spectrophotometrically (pH 6.0) at 550 mμ as described by Keilin and Hartree (12). It was preserved in solution (4.5×10^{-5} M, pH 5.0) in the ice box at 5°C. with a trace of toluene included to prevent bacterial contamination and destruction.

The manometric determinations of cytochrome oxidase activity were made with conical Warburg flasks and manometers. In all experiments, except those on effects of varying cytochrome c concentration, the main compartment of each flask received 0.5 cc. of 0.33 M glycylglycine buffer of pH 6.9 (final concentration 0.05 M), 0.5 cc. of the oxidase preparation, 1.0 cc. of 4.5×10^{-5} M cytochrome c (final concentration 1.36×10^{-5} M), and 1.0 cc. of water or a solution of the desired oxidase inhibitor in a concentration to give the final concentrations shown in the experimental section of the paper. The side arm contained 0.3 cc. of 0.22 M hydroquinone, metal-free cysteine, *p*-phenylenediamine, or sodium succinate according to the substrate desired; each substrate solution was brought to pH 6.9 before placing in the flasks. The flask center cups were left empty. For experiments concerned with effect of variation in cytochrome c concentration, the volumes were: 0.5 cc. oxidase, 0.5 cc. glycylglycine, 0 to 4.0 cc. cytochrome c solution (with water to make the total initial volume in the main compartment 5.0 cc.), and 0.5 cc. hydroquinone solution in the side arm. The flasks were equilibrated in the bath, at 20°C. unless otherwise specified, the substrate tipped in, and a preliminary period of 15 minutes allowed for minor pressure fluctuations to disappear. Readings of oxygen uptake were then made for the next 2 hours; all calculations here given are based on this 2 hour period, during which the total oxygen consumption was of the order of 50 to 200 c. mm. in the control flasks. The oxygen consumption followed a linear course under the conditions here defined, but use of higher final concentrations of the oxidase with hydroquinone as substrate led to the development of a cumulative inhibition of the enzyme by some unidentified oxidative product, possibly quinone.

The gas mixtures were prepared over water, passed through the flasks while the latter were shaken in the bath, and analyzed manometrically for oxygen by the method of Warburg and Kubowitz (13).

In extension of previous experiments with low oxygen tension, cyanide, and carbon monoxide on the respiration of the eggs, analogous experiments to determine the effect of azide on egg respiration were made by the Warburg direct method as previously described (3); the effect of hydrogen sulfide on egg respiration was determined by the method of Dixon and Keilin (14). In each case, eggs were obtained and fertilized in the usual way, concentrated by allowing to settle, then diluted to give a final egg concentration of 2 per cent by volume; in the course of this dilution a solution of 0.55 M glycylglycine in sea water at pH 8.0 (final concentration 0.05 M) was included to the extent of 10 per cent of the total volume. Each flask contained 0.5 cc. of a solution of the inhibitor solution. At 30 minutes after fertilization, 5.0 cc. of the egg suspension was added to the flasks, the temperature being maintained at 20°C. throughout.

In the course of this work on the eggs it was found, in running controls to ascertain

the division of the eggs at various hydrogen sulfide concentrations, that respiration experiments on the effect of hydrogen sulfide at pH 8 could be run without alkali in the center cup, the respiratory carbon dioxide being readily absorbed by the glycylglycine buffered sea water serving as medium for the eggs. Since the vapor pressure of carbon dioxide over sea water has in fact been found (15) to be negligible at pH values above 7.8, the method might be applied more generally for measurement of respiration under conditions where rather alkaline media can be used.

EXPERIMENTAL RESULTS

Under the conditions here employed the oxygen uptake by the cytochrome oxidase in presence of cytochrome c was linear over a 2 hour period and, with a given preparation, proceeded at nearly the same rate whether hydroquinone (0.02 M), cysteine (0.02 M), or phenylenediamine (0.02 M) was used as substrate (Fig. 1). The autooxidation of substrate in presence of heat-inactivated oxidase (100°C. for 10 minutes) and cytochrome c was, as indicated by the representative data of Fig. 1, between 5 and 10 per cent of that in the active preparation. This autooxidation was almost insensitive to the inhibitors to be mentioned below. For all subsequent discussion in the present paper, rates of oxygen uptake refer to the net values obtained by subtraction of the uptake by heat-inactivated enzyme plus cytochrome c plus substrate from the uptake by unheated enzyme plus cytochrome c plus substrate. In contrast to heart muscle (8) the eggs were readily freed, by simple washing and dialysis of the fragmented cells, of intermediate catalysts capable of causing a substantial oxygen uptake in absence of added cytochrome c (Fig. 1).

Cytochrome Oxidase Activity in Relation to Cytochrome c Concentration.—As previously shown by Stotz (8) for beef heart muscle, the activity of cytochrome oxidase from the eggs increased with the concentration of cytochrome and approached a maximum value at a concentration of cytochrome c somewhat below 10^{-4} M (Fig. 2). The cytochrome c concentration required for half activation of the oxidase was approximately 4×10^{-6} M. This value for unfertilized and fertilized *Arbacia* eggs at 20°C. may be compared with the value of approximately 6×10^{-6} M for half activation of cytochrome oxidase from beef heart muscle as determined by Stotz, Altschul, and Hogness (16) at 38°C.

Concentration of Cytochrome Oxidase in Unfertilized and Fertilized Arbacia Eggs.—When tested in the presence of excess cytochrome c the concentration of cytochrome oxidase was found to be nearly the same in unfertilized and in fertilized *Arbacia* eggs (Table I). Each unit of activity is defined, following Stotz, as 10 c.mm. oxygen uptake per hour at 20° C. Efforts to determine the activity of *Arbacia* egg cytochrome oxidase at 38°C. were invalidated by the fact that the oxidase, with hydroquinone as a substrate,

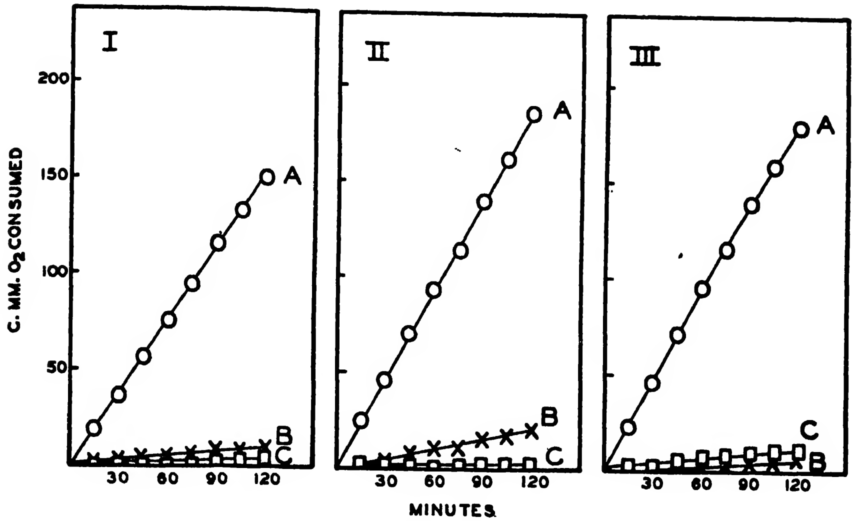

FIG. 1. Oxygen consumption at pH 6.8 by: *A*, cytochrome oxidase plus 1.36×10^{-5} M cytochrome c; *B*, heat inactivated cytochrome oxidase plus 1.36×10^{-5} M cytochrome c; *C*, cytochrome oxidase with no added cytochrome. The reductants for cytochrome c were: I, 0.02 M hydroquinone; II, 0.02 M *p*-phenylenediamine; III, 0.02 M cysteine. In this experiment all *A* and *C* samples were aliquots from the same oxidase preparation; the rates of oxidation with the various reductants may therefore be directly compared. Temperature, 20°C.

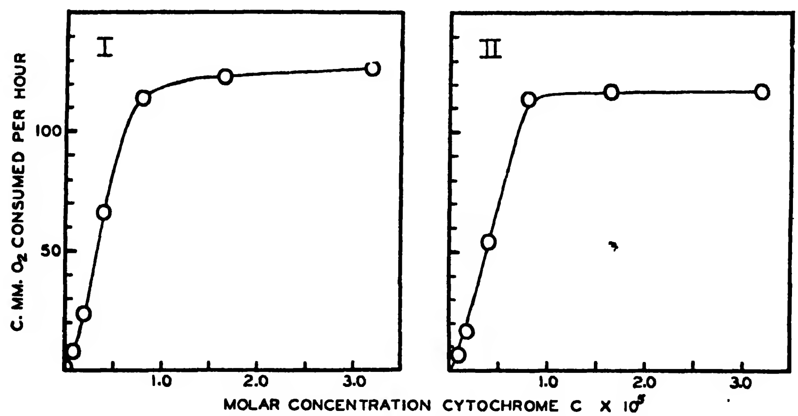

FIG. 2. Oxygen consumption at pH 6.8 by cytochrome oxidase from (I) unfertilized *Arbacia* eggs and (II) fertilized *Arbacia* eggs as a function of cytochrome c concentration with 0.02 M hydroquinone as reductant for cytochrome c. Temperature, 20°C.

was rapidly inactivated at this temperature, while the rate of autooxidation was relatively high. However, using the measurements at 20°C. with allowance for the temperature factor, the amount of cytochrome oxidase

activity present in the eggs compared favorably with that in various rat tissues, as determined by Stotz.

The Q_{O_2} values of Table I (cubic millimeters of oxygen taken up by cytochrome oxidase in 3.2×10^{-5} M cytochrome c and 0.02 M hydroquinone per hour per mg. original eggs, dry weight) may be compared with the following approximate Q_{O_2} values for the living whole eggs: unfertilized, 0.4–0.5; fertilized, 2; fertilized optimally stimulated by 4,6-dinitrocresol and other substituted phenols (1, 4) which act through the cyanide-sensitive egg respiratory system, 7–8.

Inhibition of Arbacia Cytochrome Oxidase by Carbon Monoxide.—The initial experiments with oxidase inhibitors were made to determine which

TABLE I

Analysis of Arbacia Eggs for Cytochrome Oxidase by Stotz (8) Method at 20°C. Cytochrome c, 3.2×10^{-5} M; Hydroquinone, 0.02 M

Exp. No.	Date	Cytochrome oxidase units* per mg. dry weight	
		Unfertilized eggs	Fertilized eggs
134 W	9–19–40	1.01	—
136 W	9–20–40	—	0.83
137 W	9–21–40	0.99	0.85
138 W	9–22–40	0.95	0.88

* The Q_{O_2} values corresponding to the cytochrome oxidase units may be obtained by multiplying the figures in the table by a factor of 10.

of the respiratory and division blocking agents previously (3) used for eggs could be considered to derive their physiological action from suppression of cytochrome oxidase activity. It was soon apparent, however, that the quantitative reaction of the oxidase to inhibitors was so different from that of the fertilized *Arbacia* eggs as to merit a considerable exploration of the properties of the oxidase with the hope that the resulting data might help to define the probable enzymic relationships in the living *Arbacia* eggs. The experimental results will first be presented; comparisons of the respective reactions of the eggs and the oxidase to inhibitors, together with theoretical considerations arising from the experiments with the various inhibitors, will be discussed in a separate section below, to which reference should be made for definition of terms employed in the captions accompanying the figures and tables.

The oxidase activity was strongly inhibited by carbon monoxide in the dark (Fig. 3). The inhibition was almost completely reversed (Fig. 4) by a carbon arc lamp. The relative inhibition by a given partial pressure

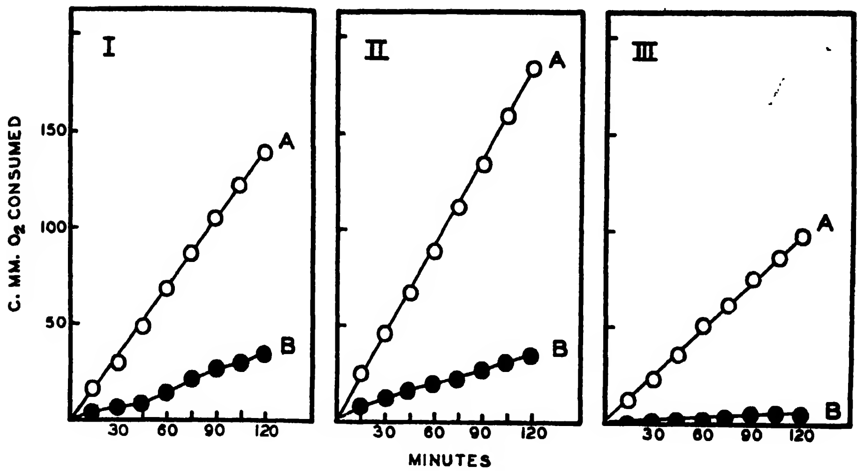

FIG. 3. Effect of carbon monoxide in the dark on oxygen consumption at pH 6.8 by cytochrome oxidase plus 1.36×10^{-5} M cytochrome c with various reductants for cytochrome c: I*A*, 0.02 M hydroquinone in air; I*B*, 0.02 M hydroquinone in 7.5 per cent O_2 − 92.5 per cent CO, *K* (See Table II) = 3.2. II*A*, 0.02 M *p*-phenylenediamine in air, II*B*, 0.02 M *p*-phenylenediamine in 6.8 per cent O_2 − 93.2 per cent CO, *K* = 1.8. III*A*, 0.02 M cysteine in air, III*B*, 0.02 M cysteine in 6.5 per cent O_2 − 93.5 per cent CO, *K* = 0.6. Temperature, 20°C.

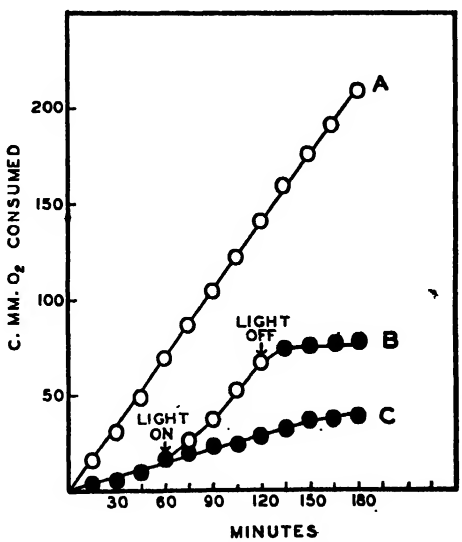

FIG. 4. Effect of carbon monoxide in the dark and in the light on oxygen consumption at pH 6.8 by cytochrome oxidase plus 1.36×10^{-5} M cytochrome c plus 0.02 M hydroquinone. *A*, control in air; *B*, in 7.5 per cent O_2 − 92.5 per cent CO, illuminated for period designated; *C*, in 7.5 per cent O_2 − 92.5 per cent CO, kept dark throughout experiment.

of carbon monoxide was apparently not greatly altered by variation in the concentration of cytochrome c or the concentration of hydroquinone used as reductant for the cytochrome c (Table II).

In experiments serving as controls for the carbon monoxide experiments, the oxidase was found to have full activity in 7 per cent oxygen–93 per cent nitrogen; it was about 5–10 per cent inhibited in 6 per cent oxygen–94 per cent nitrogen.

TABLE II

Inhibition of Arbacia Cytochrome Oxidase by Carbon Monoxide in the Dark at Two Concentrations of Cytochrome c and Two Concentrations of Hydroquinone. Temperature, 20°C.

Cytochrome c concentration	Hydroquinone concentration	$\frac{pCO}{pO_2}$	O_2 consumed in 2 hrs.		$K = \frac{n}{1-n} \cdot \frac{pCO}{pO_2}$
			CO absent	CO present	
moles per l. $\times 10^6$	*moles per l.*		*c.mm.*	*c.mm.*	
1.36	0.02	12.2	139	29	3.2
0.34	0.02	17.0	52	8	3.1
1.36	0.001	14.4	55	13	4.5

TABLE III

Inhibition of Arbacia Cytochrome Oxidase by Various Concentrations of Sodium Cyanide at Two Concentrations of Cytochrome c with Hydroquinone As Substrate and at One Concentration of Cytochrome c with p-Phenylenediamine as Substrate. Temperature, 20°C.; pH 6.9

Concentration total cyanide	O_2 consumed in 2 hrs. by oxidase with 1.36 $\times 10^{-6}$ M cytochrome c and 0.02 M hydroquinone	Inhibition	O_2 consumed in 2 hrs. by oxidase with 0.34 $\times 10^{-6}$ M cytochrome c and 0.02 M hydroquinone	Inhibition	O_2 consumed in 2 hrs. by oxidase with 1.36 $\times 10^{-6}$ M cytochrome c and 0.02 M *p*-phenylenediamine	Inhibition
moles per l. $\times 10^6$	*c.mm.*	*per cent*	*c.mm.*	*per cent*	*c.mm.*	*per cent*
0	98	0	61	0	171	0
1	57	41.9	39	36.0	73	57.2
4	29	70.4	29	52.5	50	70.7
16	14	85.6	23	62.3	41	76.0
64	10	89.8	20	67.2	31	81.9
256	0	100.0	0	100.0	4	97.5

Inhibition of Arbacia Cytochrome Oxidase by Sodium Cyanide.—The oxidase activity was strongly inhibited at very low concentrations of sodium cyanide (Table III), the logarithm of the ratio of inhibited to uninhibited respiration varying approximately linearly with the logarithm of the cyanide concentration (Fig. 5). The relative inhibition by a given concentration of cyanide was apparently not greatly altered by variation in the concentration of cytochrome c (Table III) nor by use of *p*-phenylenediamine instead of hydroquinone as substrate.

Inhibition of Respiration and Cell Division of Fertilized Arbacia Eggs and Also Arbacia Cytochrome Oxidase by Sodium Azide.—To supplement earlier experiments (3) with low oxygen tension, carbon monoxide, and cyanide,

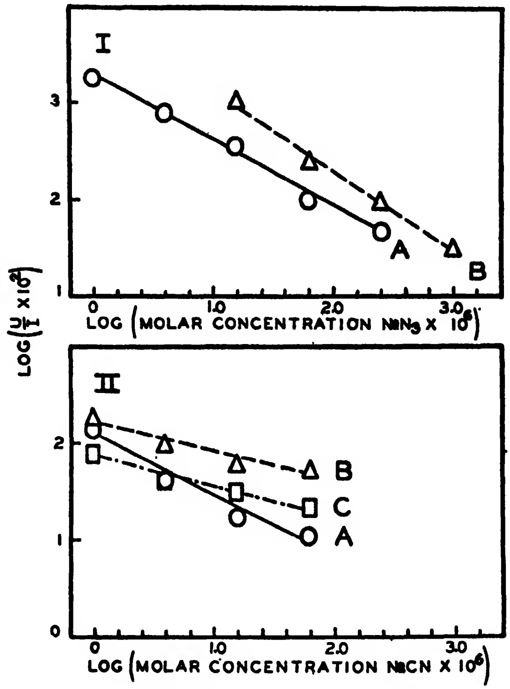

FIG. 5. Plot of log [inhibitor concentration] against log $\frac{U}{I}$ to test the equation $\frac{U}{I} = K$ [concentration inhibitor]$^{-a}$ for: I*A*, sodium azide at pH 6.8 on cytochrome oxidase plus 1.36×10^{-5} M cytochrome c plus 0.02 M hydroquinone; I*B*, sodium azide at pH 6.8 on cytochrome oxidase plus 0.34×10^{-5} M cytochrome c plus 0.02 M hydroquinone. II*A*, sodium cyanide at pH 6.8 on cytochrome oxidase plus 1.36×10^{-5} M cytochrome c plus 0.02 M hydroquinone. II*B*, sodium cyanide at pH 6.8 on cytochrome oxidase plus 0.34×10^{-5} M cytochrome c plus 0.02 M hydroquinone. II*C*, sodium cyanide at pH 6.8 on cytochrome oxidase plus 1.36×10^{-5} M cytochrome c plus 0.02 M *p*-phenylenediamine. Temperature, 20°C.

the effect of various concentrations of sodium azide on respiration and cell division of fertilized *Arbacia* eggs was determined and expressed (Fig. 6) by methods identical with those previously used for the other inhibitors. Attention is directed to two points of interest: First, approximately 50 per cent of the respiration of the fertilized eggs was insensitive to azide under the present conditions of experiment; secondly, 50 per cent inhibition

of cell division occurred at an azide concentration inhibiting respiration by only about 10 per cent; complete and reversible inhibition of cell division occurred at an azide concentration inhibiting respiration by about 50 per cent.

The cytochrome oxidase activity in the cell-free preparation was also inhibited by sodium azide (Table IV), the logarithm of the ratio of inhibited to uninhibited respiration varying linearly with the logarithm of the azide concentration (Fig. 5). The relative inhibition by a given concentration of azide was not greatly altered by variation in the concentration of cytochrome c (Table IV).

TABLE IV

Inhibition of Arbacia Cytochrome Oxidase by Various Concentrations of Sodium Azide at Two Concentrations of Cytochrome c with 0.02 M Hydroquinone as Substrate. Temperature, 20°C.; pH 6.8

Concentration total azide	O_2 consumed in 2 hrs. by oxidase with 1.36×10^{-5} M cytochrome c	Inhibition	O_2 consumed in 2 hrs. by oxidase with 0.34×10^{-5} M cytochrome c	Inhibition
moles per l. $\times 10^{5}$	*c.mm.*	*per cent*	*c.mm.*	*per cent*
0	116	0	61	0
1	110	5.2	56	8.2
4	102	12.0	56	8.2
16	90	22.4	43	29.4
64	59	49.0	28	54.0
256	38	67.1	14	77.0

Inhibition of Respiration and Cell Division of Fertilized Arbacia Eggs and Also Arbacia Cytochrome Oxidase by Sodium Sulfide.—To supplement earlier experiments (3) with other inhibitors the effect of various concentrations of sodium sulfide on respiration and cell division of fertilized *Arbacia* eggs was determined (Fig. 6). Cell division was 50 per cent inhibited at a sulfide concentration inhibiting respiration by only about 10 per cent. Complete, but *not* reversible, inhibition of cell division occurred at a sulfide concentration inhibiting respiration by about 50 per cent. At the lethal concentration of sulfide the respiration was inhibited by about 80 per cent. Owing to the lethal action of the sulfide it was impossible to determine from the present experiments whether any fraction of the vital respiration was insensitive to sulfide.

The cytochrome oxidase activity was also inhibited by sodium sulfide, complete suppression of activity being produced at approximately 1×10^{-3} M sodium sulfide at pH 6.9 (Fig. 7). Experiments of this type, though repeatedly carried out, yielded rather unsatisfactory results because, at

concentrations above 1×10^{-3} M, the sulfide itself was rapidly oxidized by the heated and still more rapidly by the unheated oxidase preparation.

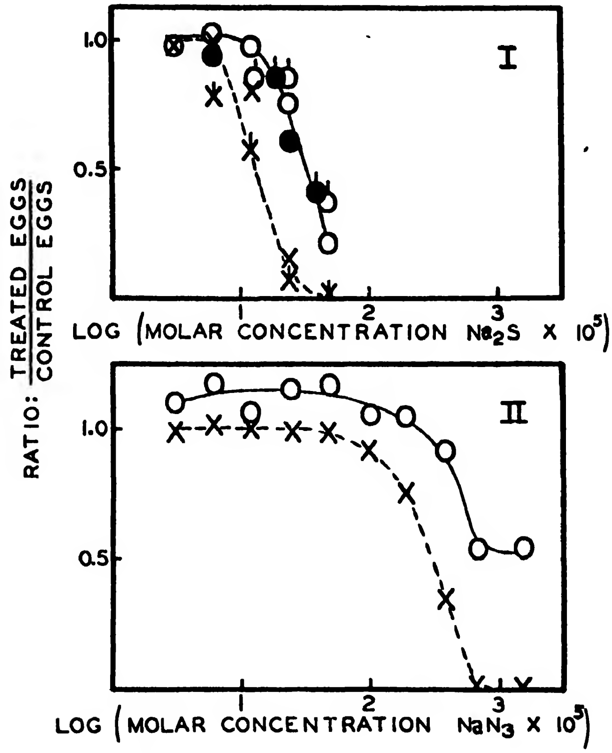

FIG. 6. Oxygen consumption and cell division of fertilized eggs of *Arbacia punctulata* in I, various concentrations of sodium sulfide at pH 7.9; II, various concentrations of sodium azide at 7.9. The reagents were added 30 minutes after fertilization. Temperature, 20°C. In Fig. 6

$$\text{O-O} = \frac{\text{O}_2 \text{ consumed in treated eggs}}{\text{O}_2 \text{ consumed in control eggs}},$$

$$\text{X-X} = \frac{\text{Cell division in treated eggs}}{\text{Cell division in control eggs}}$$

In I, the open circles represent measurements by the direct Warburg method; the solid circles represent simultaneous measurements by the Dixon-Keilin method on aliquots from the same egg sample. The plain circles refer to one experiment, the circles with bar to a second experiment on a different sample of eggs.

It is not at present clear whether this oxidation of sulfide is catalyzed by the cytochrome oxidase or by the echinochrome-protein complexes accompanying the cytochrome oxidase.

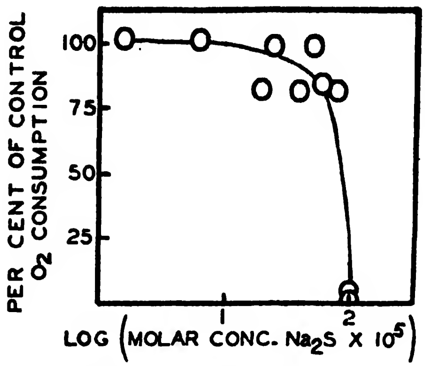

FIG. 7. Effect of various concentrations of sodium sulfide at pH 6.8 on cytochrome oxidase plus 1.36 × 10^{-5} M cytochrome c plus 0.02 M hydroquinone. Temperature, 20°C.

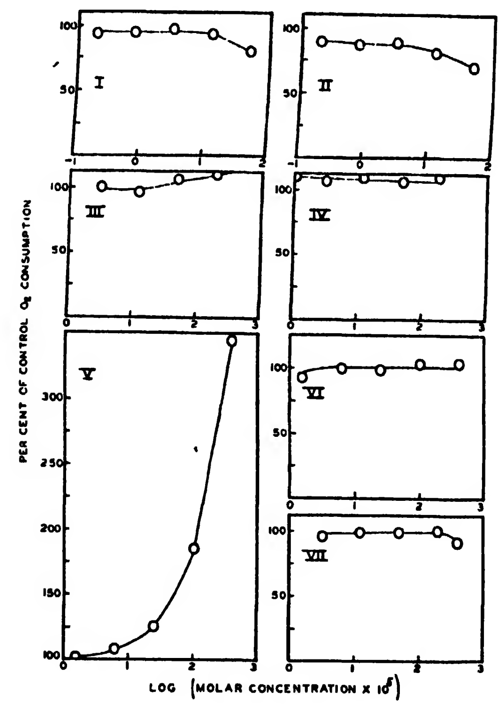

FIG. 8. Effect of various concentrations of each of seven agents at pH 6.8 on cytochrome oxidase plus 1.36 × 10^{-5} M cytochrome c plus 0.02 M hydroquinone. I, 2, 4-dinitro-*o*-cyclohexylphenol; II, 2, 4-dinitrothymol; III, 5-isoamyl-5-ethyl barbituric acid; IV, phenylurethane; V, sodium diethyldithiocarbamate; VI, iodoacetic acid; VII, 8-hydroxyquinoline.

Effect of Miscellaneous Agents on Arbacia Cytochrome Oxidase.—In view of the suggestion, made by Keilin and Hartree (17), that cytochrome oxidase may be a copper compound, the effects of two well known inhibitors of copper containing enzymes, 8-hydroxyquinoline and sodium diethyldithiocarbamate, were tested on the *Arbacia* cytochrome oxidase. Neither produced any inhibition of activity in the highest concentrations soluble in the medium used (Fig. 8). In fact, the diethyldithiocarbamate produced

TABLE V

Concentration of Sodium Cyanide, Sodium Azide, and Sodium Sulfide Required to Produce 50 Per Cent Inhibition of Respiration, Cell Division, and Cytochrome Oxidase Activity of Fertilized Arbacia Eggs. Temperature, 20°C. For Method of Calculation of Ion and Molecule Concentrations See Text and Reference 21

	Cyanide	Azide	Sulfide
	moles per l. $\times 10^6$	*moles per l.* $\times 10^6$	*moles per l.* $\times 10^6$
1. Total *extracellular* concentration of inhibitor for 50 per cent inhibition of respiration at pH 7.9	69	8200	350
2. Calculated *intracellular* concentration of inhibitor molecules at pH 6.8 corresponding to total concentration in item 1	66	5.2	39
3. Calculated *intracellular* concentration of inhibitor anions at pH 6.8 corresponding to total concentration in item 1	0.3	650	25
4. Total *extracellular* concentration of inhibitor for 50 per cent inhibition of cell division at pH 7.9	44	3200	130
5. Calculated *intracellular* concentration of inhibitor molecules at pH 6.8 corresponding to total concentration in item 4	42	2.0	15
6. Calculated *intracellular* concentration of inhibitor anions at pH 6.8 corresponding to total concentration in item 4	0.2	250	10
7. Total concentration of inhibitor for 50 per cent inhibition of cytochrome oxidase plus 1.36×10^{-5} M cytochrome c plus 0.02 M hydroquinone at pH 6.8	1.4	66	850
8. Calculated concentration of inhibitor molecules at pH 6.8 corresponding to total concentration in item 7	1.3	0.5	520
9. Calculated concentration of inhibitor anions at pH 6.8 corresponding to total concentration in item 7	0.1	66	330

a large increase in oxygen uptake in experiments with *Arbacia* egg cytochrome oxidase. This may be provisionally attributed to oxidation of the diethyldithiocarbamate by the oxidase or by the echinochrome-protein complexes accompanying the cytochrome oxidase. Keilin and Hartree (18), in a paper appearing after the completion of the present experiments, showed that diethyldithiocarbamate is oxidized to tetraethyldithiocarbamyl-disulfide by a cytochrome oxidase preparation from beef heart muscle. They also showed the latter compound to be a powerful inhibitor of succinic dehydrogenase. Incidentally, while oxygen consumption of fertilized

Arbacia eggs was affected little or not at all by sodium diethyldithiocarbamate in concentrations up to 4×10^{-3} M, the cell division was about 10 per cent inhibited at 3×10^{-5} M and 50 per cent inhibited (partially irreversibly) at 4×10^{-3} M.

To clarify the results previously obtained on living *Arbacia* eggs, a number of other physiologically active agents were employed with the oxidase (Fig. 8). With the exception of sodium chloride (not shown), which produced a complete inhibition of oxidase activity in a concentration of 0.6 M at pH 6.8, none of the agents produced a substantial inhibition of *Arbacia* cytochrome oxidase activity until concentrations greatly exceeding the physiologically active concentrations were reached; this indicates that their physiological inhibition of egg respiration and cell division is attributable to their action on enzyme systems other than cytochrome oxidase.

Quantitative Comparison of Effect of Various Agents on Egg Respiration, Egg Cell Division, and Cytochrome Oxidase.—The concentrations of sodium cyanide, sodium azide, and sodium sulfide required to produce 50 per cent inhibition of fertilized *Arbacia* egg respiration, fertilized *Arbacia* egg cell division, and *Arbacia* cytochrome oxidase have been assembled (Table V) from the data of this and a previous paper (3).

In comparing the effects of these agents on the eggs and on the oxidase, it should be noted that cyanide and azide apparently penetrate fertilized *Arbacia* eggs only as undissociated molecules (19) and that the form of each of these agents which enters into complexes with metalloporphyrins may well be the anion (20). On the basis of theoretical considerations detailed elsewhere (21) the probable concentrations of anions (CN^-, N_3^-, and HS^-) and of undissociated molecules (HCN, HN_3, and H_2S) in the aqueous phase of the egg cytoplasm have been calculated (Table V), using pK′ values (22) of 9.2, 4.7, and 7.0 for hydrogen cyanide, hydrogen azide, and hydrogen sulfide (first hydrogen).

It has recently been shown by Fisher and Öhnell (23) that the effects of cyanide on a number of physiological processes conform to the equation $\frac{U}{I} = K[CN]^{-a}$, where U is the fraction of function uninhibited, I is the fraction of function inhibited, $[CN]$ is the molar total cyanide concentration in the medium, and K and a are constants. As an empirical approach, while postponing discussion of the probable significance of such numerical values until the mechanism of the inhibitor action is better understood, log [inhibitor concentration] has been plotted against log $\frac{U}{I}$ for cyanide and azide inhibition of cytochrome oxidase (Fig. 5). In these plots the

experimental points, though not accumulated specifically to test this possibility, are found to conform approximately to a straight line, as demanded by the above equation.

Attempts to Isolate Cytochrome c from Fertilized Arbacia Eggs.—Previous qualitative spectroscopic examination of brei from fertilized *Arbacia* eggs after reduction with sodium hydrosulfite failed (3) to reveal the presence of cytochrome c. These experiments have now been extended by repeated attempts to isolate cytochrome c by the method of Stotz (8). The resultant products displayed no specific light absorption at 550 mμ and were devoid of cytochrome c activity when examined manometrically by the Stotz method; control experiments in which known amounts of cytochrome c were carried through the testing process showed that cytochrome c, if present, could have been detected in concentrations down to approximately 2 micrograms per gram of wet fertilized *Arbacia* eggs. On the basis of these data and those of Fig. 2, it seems safe to conclude that cytochrome c cannot carry a significant fraction of the oxygen consumption of fertilized *Arbacia* eggs.

Also in extension of previous experiments (5, 7) by the Thunberg method, it has been found manometrically that the cytochrome oxidase preparation has no succinic dehydrogenase activity; succinate, in a final concentration of 0.02 M at pH 6.8 and 20°C., caused no extra oxygen uptake when added to *Arbacia* cytochrome oxidase saturated with added cytochrome c.

DISCUSSION

The present experiments were undertaken with a view to establishing a basis from which the effects of carbon monoxide, cyanide, azide, and sulfide on fertilized *Arbacia* eggs could be used to clarify the mechanism by which energy from oxidative processes is utilized for the support of cell division in such eggs. A number of facts relevant to this objective have been established.

1. The eggs of *Arbacia punctulata* contain an enzyme capable of oxidizing reduced cytochrome c.

2. The amount of the enzyme, as measured by means of its activity toward cytochrome c as a representative substrate, is more than sufficient to account for the highest rate of oxygen utilization yet observed in the intact, living fertilized eggs.

3. In its rapid reaction with molecular oxygen, its light reversible inhibition by carbon monoxide, and its inhibition by cyanide, azide, and sulfide—but not by agents forming complexes with copper—the enzyme displays

properties which are those of an electromotively active iron-porphyrin compound.

4. The enzyme, when acting with cytochrome c as substrate, is completely inhibited by cyanide or azide, just as is the cell division of the living fertilized *Arbacia* eggs. In contrast, the respiration of the fertilized egg can be inhibited to a maximum of only about 70–80 per cent by cyanide and only about 50 per cent by azide.

5. In the equation $K = \frac{n}{1\text{-}n} \cdot \frac{p\text{CO}}{p\text{O}_2}$, where n is fraction of respiration not inhibited, and pCO and $p\text{O}_2$ are the carbon monoxide and oxygen partial pressures, the apparent values of the inhibition constant for the action of carbon monoxide upon the enzyme in the dark were found to be in the range of 0.5 to 5, depending on the concentration of cytochrome c and the nature and concentration of the reductant for cytochrome c. The corresponding value for the fertilized *Arbacia* eggs is very much larger than this, being of the order of 60 on the assumption that the respiration is completely sensitive to carbon monoxide.

6. Previous qualitative observations regarding the low concentration, or absence, of cytochrome c in the eggs are confirmed; further quantitative observations place the highest possible concentration of cytochrome c at a level too low to be of any probable significance for the respiration of fertilized *Arbacia* eggs.

When considered in conjunction with what is now known regarding the combination of cyanide and other nitrogenous materials with iron-porphyrin compounds, the present results appear to be of some potential significance, not only for respiration of *Arbacia* eggs, but in explaining the action of cyanide on cell respiration in general.

It was shown by Barron (24) that cyanide forms electromotively active hemochromogens when added to certain iron-porphyrin compounds. The oxidation-reduction potentials (E_0' values at pH 7.0) of such cyanide hemochromogens are, other factors being equal, much lower (100–300 mv. or more) than those of hemochromogens containing the same iron-porphyrin nucleus with other simple nitrogenous bases or proteins substituted for cyanide in the complex. These observations have recently been extended by Davies (20) and the theory has been developed in detail by Clark, Taylor, Davies, and Vestling (25). Continuing his previous work (26) on the parallelism between oxidation-reduction potential difference between catalyst and substrate system on the one hand and catalytic activity on the other, Barron (27) also showed that various hemochromogens could act as oxidative catalysts if supplied with a substrate system having a potential at an appropriate level below that of the hemochromogen.

In the light of these findings, it appears that the action of cyanide in inhibiting cell respiration may be interpreted on the hypothesis that the cyanide forms, with the iron-porphyrin of the cytochrome oxidase, a complex having a potential lower than that of the original oxidase. Whether or not the cyanide complex can then continue in part the function of the oxidase will depend, among other factors, on the potentials of the catalytic systems next lower in the oxidative chain. For example, if this system were exclusively cytochrome c (E_0' at pH 7, + 257 mv. (29)), the cyanide complex (E_0' from pH 5 to 8, −183 mv. for the cyanide complex with blood hemin) could not serve as catalyst and the respiration of the cell would be completely inhibited. On the other hand, if a catalyst with a potential somewhat below that of cytochrome c, and near or suitably below that of the cyanide hemochromogen were available, the cell would retain some capacity to consume oxygen, with cyanide hemochromogen partially substituting for the oxidase.[1] On this basis, the residual respiration displayed by certain cells in the presence of cyanide would remain, in certain instances at least, a metal-catalyzed oxidation and not, as hitherto implicitly supposed, a catalysis carried on by metal-free systems.

With this background in mind, a number of observations regarding the effects of cyanide and analogous inhibitors on the respiration and cell division of *Arbacia* eggs may possibly be given a provisional qualitative explanation on the basis of an assumption derived, by analogy, from the experimental data on cyanide referred to above. This assumption is that the inhibitors cyanide, azide, carbon monoxide, and possibly sulfide, change the state of intracellular binding of the iron-porphyrin which initially functioned as part of the enzyme acting as the terminal link at the oxygen end of the respiratory chain; as a result of the change the potential of the iron-porphyrin in its new linkage is lower than in the untreated cell. While the data necessary for the further elaboration of this assumption are not at present available it may be noted that both the ability of any given reagent to enter into complex formation with the iron-porphyrin and the magnitude of the potential shift obtainable with any given inhibitor

[1] The hypothesis here proposed regarding the mechanism of action of cyanide on cell respiration is considered by the authors to be an extension, to living systems, of the ideas developed by Barron for purely chemical systems. The possibility that cyanide (and other analogous) hemochromogens might act as physiological oxygen transfer catalysts was, so far as the authors are aware, first explicitly stated by Dr. E. G. Ball and one of the authors (M. E. Krahl) during a discussion of an evening paper presented at the Marine Biological Laboratory, Woods Hole, on July 18, 1939. The hypothesis has been further discussed by Ball (28) elsewhere.

depend, among other factors, on the particular structure of the porphyrin component.

It was observed that the vital respiration of fertilized *Arbacia* eggs was about 20 per cent insensitive to cyanide and about 50 per cent insensitive to azide. On the basis of the above hypothesis, this would be at least in part attributable to the difference in potential and in catalytic activity between the cyanide and azide iron-porphyrin complexes under the conditions operative in the *Arbacia* egg.

With each of the inhibitors dealt with in this paper it was observed that, at critical concentrations of the inhibitor, cell division was inhibited relatively more than respiration, finally being completely suppressed at inhibitor concentrations which allowed a substantial portion of the respiration to proceed. On the basis of the general hypothesis advanced above, this is the result to be expected if the potential of the particular carrier by which that fraction of the electron transfer critical for cell division is keyed to the oxidase is relatively closer to that of the oxidase than the potentials of other carriers responsible for the bulk of the overall respiration. If confirmed by independent methods of investigation this suggestion provides a partial answer to the principal question posed at the start of the investigation; it may help to define the type, and specify the potentials of, certain of the oxidative catalysts which make energy available for the cell division cycle.

It was observed that the carbon monoxide inhibition constant was much larger for the fertilized eggs than for the cell-free cytochrome oxidase-cytochrome c system. This is what would be expected if the substrate for the oxidase in the eggs had a potential substantially below that of cytochrome c; since the eggs, as shown above, appear to contain no cytochrome c, this possibility is open.

It has previously been observed that the respiration of unfertilized *Arbacia* eggs is completely insensitive to carbon monoxide (30) and is relatively less sensitive to cyanide than that of fertilized *Arbacia* eggs (31). On the basis of the general hypothesis advanced above, this means that the increase of respiration on fertilization is concerned with the entrance of a carrier system having a potential higher than those operative before fertilization, establishing a better relationship between oxidase and carrier before poisoning but, at the same time, providing an unfavorable relationship of potential after addition of carbon monoxide or cyanide.

It is suggested that this may also be the explanation for the fact that the endogenous respiration of certain cells in absence of substrate is insensitive to carbon monoxide, the respiration of the same cells becoming carbon monoxide sensitive on addition of substrate.

It has been observed that the respiration of a number of tissues (32) is sensitive to cyanide but not to azide. This apparent paradox is immediately resolved, by the present hypothesis, on two grounds: first, the relative abilities of cyanide and azide to combine with the iron-porphyrin group in question and, secondly, the respective potentials of the resulting complexes with reference to the next available carrier in the respiratory chain.

It has been observed (4) that various substituted phenols alter the sensitivity of fertilized *Arbacia* egg respiration to cyanide. With the substituted phenol alone, without cyanide, the rate of oxygen consumption of the eggs rose to an optimum as the concentration of the substituted phenol was progressively increased; at still higher concentrations the rate of oxygen consumption fell below this optimum and, at sufficiently high concentrations, below the normal. Suboptimum respiratory-stimulating concentrations of the substituted phenol induced a respiration relatively more sensitive than the normal to cyanide. Greater than optimum concentrations of the phenol caused the sensitivity to cyanide to fall at first toward the normal and then to become less sensitive to cyanide than the normal respiration. This is the course of events to be expected if the substituted phenol brings into play, at low and suboptimum concentrations, a carrier system reacting directly with the oxidase, with high and greater than optimum concentrations blocking or reversing this process and finally bringing into play considerable amounts of a carrier having a potential somewhat lower than those operating in the egg untreated with the substituted phenol.

It is proposed to conduct further experiments to throw light on this, at present, somewhat speculative theory. The theory is advanced only because of the large number of hitherto puzzling facts which it appears to correlate and because of the numerous interesting experimental suggestions to which it gives rise.

SUMMARY

1. An enzyme capable of oxidizing reduced cytochrome c (*i.e.* a cytochrome oxidase) has been obtained from *Arbacia* eggs. In 0.02 M hydroquinone, the cytochrome oxidase was half activated at a cytochrome c concentration of approximately 4×10^{-6} M. The concentration of the cytochrome oxidase was found to be nearly the same in unfertilized and fertilized eggs, the amount of the enzyme—as measured by means of its activity toward cytochrome c as a representative substrate—being more than sufficient to account for the highest rate of oxygen utilization yet

observed in the intact, living, fertilized eggs, and of the same order as that in certain rat tissues.

2. The *Arbacia* cytochrome oxidase was strongly inhibited by carbon monoxide in the dark, the inhibition being almost completely reversed by light. The inhibition constant was not greatly altered by variation in the concentration of cytochrome c or the concentration of hydroquinone used as reductant for the cytochrome c, having a value of 3 to 5 under the conditions used. The inhibition constant was about 2 with *p*-phenylenediamine as reductant for the cytochrome c, but apparently had the surprisingly low value of about 0.5 with 0.02 M cysteine as reductant.

3. The cytochrome oxidase was completely inhibited by sufficiently high concentrations of sodium cyanide, sodium azide, and sodium sulfide. It was also completely inhibited in 0.6 M sodium chloride. It was not inhibited by two inhibitors of copper containing enzymes, 8-hydroxyquinoline and sodium diethyldithiocarbamate. It was also not significantly inhibited by 2,4-dinitrothymol, 2,4-dinitro-*o*-cyclohexylphenol, phenylurethane, 5-isoamyl-5-ethylbarbituric acid, or iodoacetic acid.

4. Quantitative examination of the fertilized eggs showed that cytochrome c, if present at all, occurred in a concentration of less than 2 micrograms per gram of wet fertilized *Arbacia* eggs. On the basis of these data and those of Fig. 2, above, it seems safe to conclude that cytochrome c cannot carry a significant fraction of the oxygen consumption of fertilized *Arbacia* eggs.

It was also found that, in contrast to similar preparations from certain other animal tissues, the *Arbacia* cytochrome oxidase preparation displayed no succinic dehydrogenase activity when tested manometrically in the presence of excess cytochrome c.

5. Extending previously reported (3) experiments with other inhibitors, the effects of sodium azide and sodium sulfide on the respiration and cell division of fertilized *Arbacia* eggs were determined, the eggs being initially exposed to the reagents 30 minutes after fertilization at 20°C. With either reagent cleavage was completely blocked by a concentration of reagent which reduced the respiration to approximately 50 per cent of the normal level.

6. On the basis of certain theoretical considerations regarding the possible mechanism of action of cyanide and other respiratory inhibitors it is suggested that a fraction of the respiration apparently concerned with supplying energy for division processes in the fertilized *Arbacia* egg may be keyed into the respiratory cycle through a carrier having a somewhat higher potential than those which carry the larger portion of the egg respiration.

The theory is also employed in an effort to resolve a number of hitherto apparently paradoxical observations regarding the effects of cyanide, azide, and carbon monoxide on cell respiration.

BIBLIOGRAPHY

1. Clowes, G. H. A., and Krahl, M. E., *J. Gen. Physiol.*, 1936, **20,** 145.
2. Krahl, M. E., and Clowes, G. H. A., *J. Gen. Physiol.*, 1936, **20,** 173.
3. Clowes, G. H. A., and Krahl, M. E., *J. Gen. Physiol.*, 1940, **23,** 401.
4. Krahl, M. E., and Clowes, G. H. A., *J. Gen. Physiol.*, 1940, **23,** 413.
5. Ball, E. G., and Meyerhof, B., *J. Biol. Chem.*, 1940, **134,** 483.
6. Korr, I., Discussion to paper by H. Shapiro, Some functional correlatives of cellular metabolism, in Cold Spring Harbor symposia on quantitative biology, Cold Spring Harbor, Long Island Biological Association, 1939, **7,** 406.
7. Ballantine, R., *J. Cell. and Comp. Physiol.*, 1940, **16,** 39.
8. Stotz, E., *J. Biol. Chem.*, 1939, **131,** 555.
9. Shapiro, H., *Biol. Bull.*, 1935, **68,** 363.
10. Harvey, E. N., *Biol. Bull.*, 1931, **61,** 273.
11. McClendon, J. F., *Am. J. Physiol.*, 1909, **23,** 460.
12. Keilin, D., and Hartree, E. F., *Proc. Roy. Soc. London, Series B*, 1937, **122,** 298.
13. Warburg, O., and Kubowitz, F., *Biochem. Z.*, Berlin, 1928, **202,** 387.
14. Dixon, M., Manometric methods, Cambridge University Press, 1934, 96-110.
15. Moberg, E. G., Greenberg, D. M., Revelle, R., and Allen, E. C., *Bull. Scripps Inst. Oceanography*, 1934, **3,** 231.
16. Stotz, E., Altschul, A. M., and Hogness, T. R., *J. Biol. Chem.*, 1938, **124,** 745.
17. Keilin, D., and Hartree, E. F., *Nature*, 1938, **141,** 870.
18. Keilin, D., and Hartree, E. F., *Proc. Roy. Soc. London, Series B*, 1940, **129,** 277.
19. Krahl, Clowes, and Keltch, unpublished experiments, 1938–40.
20. Davies, T. H., *J. Biol. Chem.*, 1940, **135,** 597.
21. Krahl, M. E., and Clowes, G. H. A., *J. Cell. and Comp. Physiol.*, 1938, **11,** 1, 21.
22. Hodgman, C. D., Handbook of chemistry and physics, Cleveland, Chemical Rubber Publishing Co., 21st edition, 1936, 955.
23. Fisher, K. C., and Öhnell, R., *J. Cell. and Comp. Physiol.*, 1940, **16,** 1.
24. Barron, E. S. G., *J. Biol. Chem.*, 1937, **121,** 285.
25. Clark, W. M., Taylor, J. H., Davies, T. H., and Vestling, C. S., *J. Biol. Chem.*, 1940, **135,** 543.
26. Barron, E. S. G., and Hoffman, L. A., *J. Gen. Physiol.*, 1930, **13,** 483.
27. Barron, E. S. G., The role of iron-porphyrin compounds in biological oxidations, in Cold Spring Harbor symposia on quantitative biology, Cold Spring Harbor, Long Island Biological Association, 1939, **7,** 406.
28. Ball, E. G., Discussion published as part of reference 27.
29. Stotz, E., Sidwell, A. F., Jr., and Hogness, T. R., *J. Biol. Chem.*, 1938, **124,** 11.
30. Runnström, J., *Biol. Bull.*, 1935, **68,** 327.
31. Korr, I., *J. Cell. and Comp. Physiol.*, 1937, **10,** 461.
32. Stannard, J. N., The mechanisms involved in the transfer of oxygen in frog muscle, in Cold Spring Harbor symposia on quantitative biology, Cold Spring Harbor, Long Island Biological Association, 1939, **7,** 394.

A NEW FORM OF DIFFRACTOMETER

By RICHARD T. COX and ERIC PONDER

(*From The Biological Laboratory, Cold Spring Harbor, Long Island*)

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The results of the diffractometer method for measuring the mean diameter of red cells, originally described by Young in 1813 and rediscovered by Pijper in 1919, have hitherto been unsatisfactory for one or another of two reasons, or for both. Pijper (1919), Berganzius (1921), and Millar (1926) used white light, and in the clinical instruments of Emmons, Eve, and Haden the source is again either daylight or an electric bulb. Under such circumstances the wave length corresponding to any colored ring is unknown, *e.g.* the innermost ring is *red*, and corresponds, not to a red maximum, but to a blue-violet minimum (Allen and Ponder, 1928). Although Allen and Ponder showed that the diffraction equations are exact if monochromatic light is used, the apparatus required (a monochromator in addition to the diffractometer) is too complicated for ordinary work; most of the investigations since 1930 have accordingly been carried out by Ponder and Saslow's method (1931) in which measurements are made with respect to the junction of two colored rings. Again the wave length is unknown, and the calculations involve a constant obtained by previous calibration with monochromatic light. Add to these difficulties as regards wave length the fact that in all the methods the measurements are subjective (and the intensities which the eye has to observe are very low), and it will be appreciated that the diffractometer method is not all it might be.

The following method avoids these difficulties.

If plane monochromatic light of wave length λ is incident normally on an opaque disk of diameter d, there are produced by diffraction secondary waves having maxima and minima in directions at angles θ with the incident ray such that

$$z/\pi\lambda = d \sin \theta \qquad (1)$$

where z/π has the values 1.22 and 2.23 for minima of the first and second orders and the values 1.63 and 2.68 for maxima of the same orders. If the light falls on a number of such disks in the same orientation and randomly distributed in a plane, the intensity of the diffracted ray in any given direction will be the sum of the intensities of the rays diffracted in that direction

by all the disks separately. If the diffracted light from all the disks pass through a lens, there will be formed circular maxima and minima in the

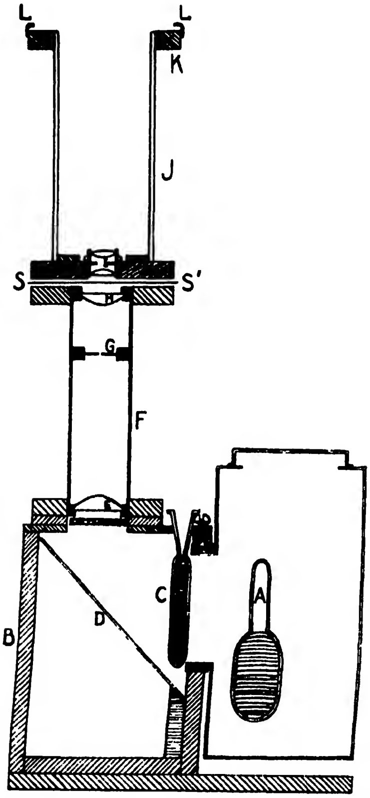

FIG. 1. Diagram of diffractometer. For details, see text

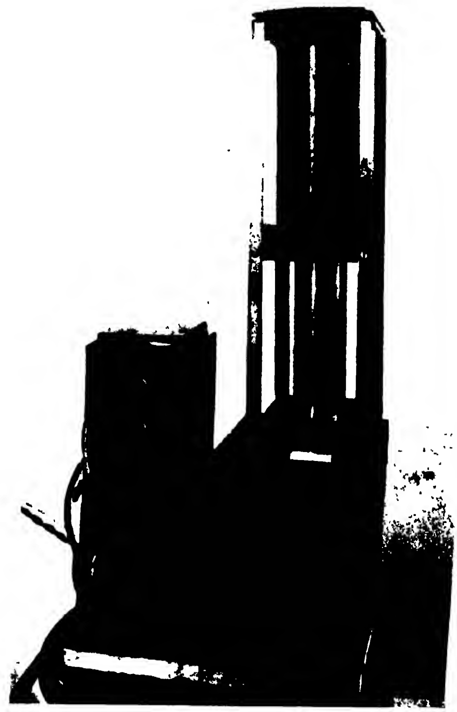

FIG. 2. The diffractometer and Hg arc

focal plane of the lens. If r is the radius of the circle corresponding to the angle of diffraction θ, then

$$\sin\theta = \frac{r}{\sqrt{r^2 + f^2}} \tag{2}$$

where f is the focal length of the lens.

The diffractometer is shown in Figs. 1 and 2. The plane SS′ of the diffracting bodies divides the instrument into two parts. The part below the plane serves to provide plane monochromatic light of convenient intensity for visual and photographic observation. The part above the plane is a camera for obtaining the diffraction pattern either on ground glass or a photographic plate.

Light from the mercury arc A passes through a circular window in the box B and a heat-filter C, which is a flat-sided flask full of water, to fall on a mirror D and to be reflected vertically through another circular window in the top of the box B. Above this window is a slot in which moves an opaque shutter and two filters, Didymium glass (Corning 512) and Wratten G, which can be placed over the window. Above the window the light is converged by the lens E set in the lower end of the opaque tube F. This lens forms a reduced image of the arc on the diaphragm G. The diaphragm is made of copper foil and has a hole 0.5 mm. in diameter at its center. Above the diaphragm, in the upper end of the tube F, is the lens H with its principal focus accurately on the pinhole in the diaphragm G. The light emerging from H is thus plane. It is almost entirely monochromatic (at least 99 per cent), giving only the green line of the mercury arc. The blood film or other diffracting system is inserted in the space SS′ above the lens H. Above this is the camera. Its parts are the compound lens I, the opaque tube J, and the top K, to which are attached rails L to hold a photographic plate holder or a piece of ground glass. The lens I is at such a distance below the top of the camera that it focuses parallel rays on the ground glass or photographic plate. The tubes F and J are blackened on the inside.

The calibration requires a knowledge of the focal length f of the lens I. This is conveniently measured by using as the diffracting system at SS′ a transmission diffraction grating. A replica grating with 7630 lines per inch is convenient for an apparatus of the dimensions shown. The image of the first order is formed at the angle θ such that

$$\lambda = D \sin \theta \qquad (3)$$

where D is the grating space. D and λ being known, θ may be computed. The distance R from the central image to the maximum may be measured on the photographic plate. The focal length may then be found by the relation

$$\tan \theta = R/f \qquad (4)$$

In our instrument, $f = 18.1$ cm.

The materials used in the apparatus are quite inexpensive. None of the optical surfaces below the diaphragm G need be of any quality, since the pinhole at G acts as a point source regardless of the shape of the wave front incident on it. The lenses H and I should be fairly well corrected for astigmatism, but they require no chromatic correction, since the light is monochromatic. In the construction of the equipment only moderate

accuracy is required. The instrument once set up is permanently in adjustment.

The plates we use are Eastman Kodak astronomical plates sensitized to the green line (Type I-G and III-G), developed for 5 minutes in D-19 developer at 65° F., washed 2 or 3 minutes in running water, and then fixed for 10 minutes. These plates are very fast, so that an exposure of 5 seconds or less is sufficient. The optics of the apparatus are so arranged that the first minimum and the first maximum fall nicely within the exposed area of the plate (Fig. 3).

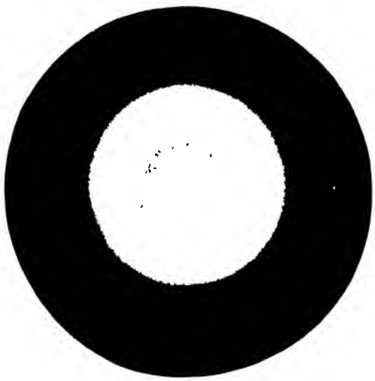

FIG. 3. A typical diffraction pattern obtained for human red cells in their spherical form. The first spectrum image of the green line of the Hg arc (the two symmetrically placed spots) appears superimposed on the diffraction pattern, of which the first minimum and the first maximum can be clearly seen.

The essentials of the diffraction patterns can be found with a very simple form of analyzer (Fig. 4). A Lange thermopile (supplied by Pfaltz and Bauer, New York City) replaces the ocular of a microscope with a moving stage in which the plate is held. The thermopile is connected to a galvanometer (enclosed lamp and scale type, sensitivity about 0.015 microamp./mm.), with a decade box used as a shunt. The microscope is illuminated by a ribbon-filament lamp, and the objective is a high dry (about 40 ×) scanning areas of about 30 μ in diameter.

Since there is considerable halation round the image of the pinhole, it is difficult to find the center from which r is to be measured. We accordingly make the red cell preparations (red cells in lecithinated plasma; *i.e.*, in the spherical form) *on the back of the diffraction grating*, covering the preparation with a large coverglass. In this way the two spots which represent the first order spectrum of the green line appear symmetrically placed about the central spot, and superimposed on the diffraction pattern of the cells. The

distance between the spots is measured, and this, divided by 2, gives the center of the plate. The horizontal transit of the moving stage is then moved outwards in the direction of the first minimum, and the reading at which the galvanometer reverses its direction is observed (*e.g.* 20 mm. on

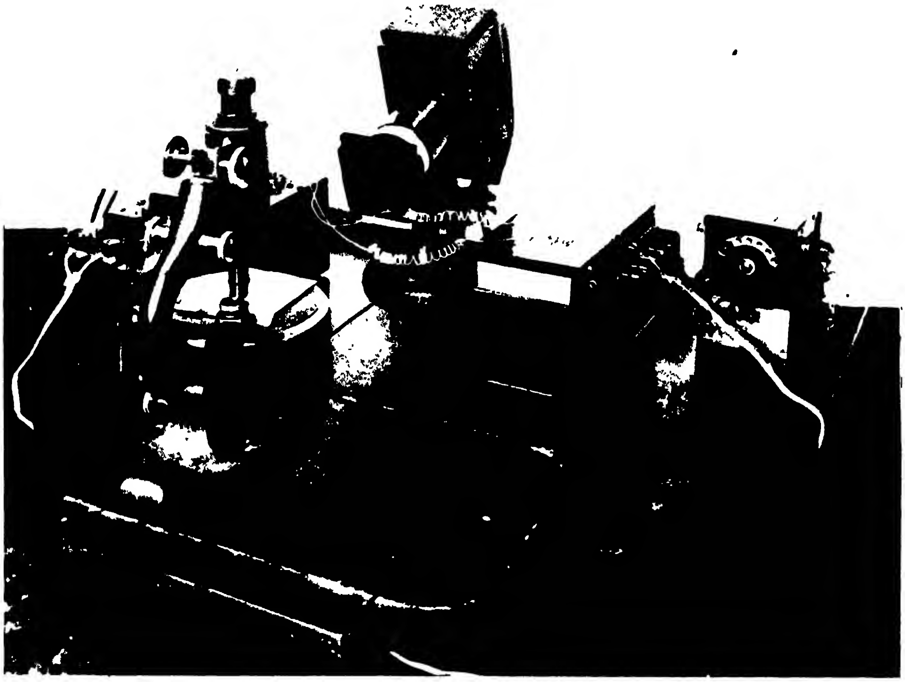

FIG. 4. The simplified microphotometer assembled

TABLE I

	Best value μ^3	Diffractometric values μ^3				
Man	87±7	80	91	83	82	85
Rabbit	57±7	54	50	57	60	56
Sheep	30±3	29	33	31	34	27

the galvanometer scale). A point on the galvanometer scale, a few millimeters less than this, is selected arbitrarily (*e.g.* 17 mm.), and by moving the horizontal transit first to the outside and then to the inside of the first minimum, two readings on the transit are obtained at which the galvanometer comes to rest at the selected point. The distance between the two transit readings, divided by 2 and added to the lower reading, gives the position of the darkest part of the first minimum by interpolation. From

this must be subtracted the reading on the transit for the central spot, and this gives r. Since we know f, λ, and z/π, d can be calculated from (1) and (2).

The severest test to which the method can be put is to compare the red cell *volume*, calculated from the diffractometrically measured radius of the cells as spheres, with the "best value" for volume obtained by other methods (hematocrit, photographic, and colorimetric methods (see Ponder, 1934). Table I shows what the correspondence is. It gives the best value and five values, found by diffraction, for the volumes of the red cells of man, the rabbit, and the sheep.

Considering that, in using the microphotometer as described, a change of 0.1 mm. on the transit corresponds to a change of about ± 5 per cent in the calculated cell volume, the averages of the five diffractometric values (man, 84, rabbit, 55, and sheep, 31) agree quite well with the best values. The apparatus may, of course, be used to give the diffraction patterns of circular objects other than red cells, or, if a slit is substituted for the pinhole G, for obtaining the diffraction patterns of hair, silk, wires, etc.

SUMMARY

A simple diffractometer is described, in which monochromatic light is focused on a pinhole, rendered parallel, and passed through a film of red cells or other objects the size of which is sought. The diffraction patterns are photographed on special plates, and the positions of the first minimum and of the first maximum are subsequently found by the use of a simplified microphotometer. The method gives substantially the same results for red cell radius and (calculated) volume as do other standard methods.

REFERENCES

Pijper, A., *South African Med. Rec.*, 1919, **27,** 8.

Berganzius, F. L., *Arch. ges. Physiol.*, 1921, **192,** 118.

Millar, W. G., *Proc. Royal Soc. London, Series B*, 1926, **99,** 264.

Allen, A., and Ponder, E., *J. Physiol.*, 1928, **66,** 37.

Ponder, E., and Saslow, G., *J. Physiol.*, 1931, **73,** 267.

Ponder, E., The mammalian red cell and the properties of hemolytic systems, Berlin, Gebruder Borntrager, 1934.

THE SIMPLEX FLICKER THRESHOLD CONTOUR FOR THE ZEBRA FINCH

By W. J. CROZIER and ERNST WOLF

(*From the Biological Laboratories, Harvard University, Cambridge*)

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I

The quantitation of the visual performance of birds presents a number of theoretically attractive possibilities; it has also presented certain technical difficulties, which undoubtedly have been responsible in part for the fact that little information on this subject has been recorded. Diurnal birds are said to have in the retina a receptor population largely or exclusively of cones (*cf.* Menner, 1929), just as such typically nocturnal birds as the owls exhibit a considerable or exclusive preponderance of retinal rods (Rochon-Du Vigneaud, 1919; *cf.* Verrier, 1939). In our examination of responses to visual flicker we have been anxious to deal with the behavior of certain possibly typical birds. The reasons for this have not been entirely, or even decisively, determined by the requirements of the doctrine of visual duplexity in vertebrates generally. We have already pointed out in other connections (Crozier and Wolf, 1938, 1939) that when a duplex curve of visual performance is found to be associated with the presence of a retinal population containing both rods and cones one is constrained to describe the two branches of the duplex performance contour by means of the parameters of their descriptive functions, rather than to associate them with intrinsic properties of "rods" and of "cones" as causative categories; this position is confirmed by the examination of the visual performance of vertebrates possessing only one general histological class of photoreceptor cells (Crozier, Wolf, and Zerrahn-Wolf, 1938; 1938–39 *a*; Crozier and Wolf, 1938–39, 1940–41).

In another direction the occurrence of simplex performance contours, when not complicated by purely accessory structural conditions, permits a test of the nature of the analytical function really usable for the description of such data (*cf.* Crozier, Wolf, and Zerrahn-Wolf, 1938–39 *a*, *b*; Crozier and Wolf, 1938–39 *b*, 1940–41). In most vertebrates the occurrence of visual duplexity restricts the usable range of the data for any really significant test by curve-fitting. This is due to the overlapping of the two

populations of sensory effects customarily attributed to the activation of rods and of cones respectively, and to the nature of the interaction between these (Crozier and Wolf, 1939–40; 1940–41 *b*). The discovery of additional cases in which a simplex visual performance curve can be demonstrated has thus a number of theoretically useful aspects.

Furthermore, since the visual acuity of birds is in general notably high one could expect that the performance contour would in general be pitched at a comparatively low intensity. This should make possible certain extensions of investigations otherwise hampered by the fact that, ordinarily, high intensities of illumination are difficult to manipulate precisely for the reasonably complete measurement of reaction contours, and if required to be monochromatic such intensities are difficult or even impossible to obtain for experiments of this kind.

The most generally applicable procedure for the investigation of visual capacity in diverse animals is unquestionably that based upon response to flicker. The great majority of animals with image-forming eyes give forced reactions to moving patterns in the visual field, provided the rate of the movement and the luminous intensities of the parts of the pattern are suitably adjusted. Most birds, with the possible exception of owls (*cf.* Bartels, 1931), exhibit eye nystagmus to moving patterns. The head nystagmus in doves and pigeons is well known (*cf.* Visser and Rademaker, 1934; Mowrer, 1936). For the quantitation of this performance the procedure we have used with various other animals (*cf.* Crozier and Wolf, 1940–41 *a*) requires a sufficiently small, reactive bird. We have used the Australian zebra finch (*Taeniopygia castanotis* (Gould)). The retina of passerine birds, for example the sparrows, is usually described as either exclusively of cones, or quite predominantly so constituted (*cf.* Menner, 1929; Slonaker, 1918). Our study of the zebra finch retina shows it to be devoid of rods. The eye has a pecten of good size. The possible significance of the pecten will be considered in connection with other experiments.

II

The zebra finches used for the observations were obtained from Dr. Roy M. Whelden of this Laboratory, who has raised several generations of this stock. Four males were employed throughout. They proved to be a decidedly homogeneous group, reactively. When surrounded by a revolving striped cylinder head nystagmus is easily recognizable. The head turns in the direction of the stripe motion, often through more than 180°, then rapidly returns to the initial direction; so long as the rotation speed is low enough, or the illumination high enough, these motions are regularly repeated. With sufficiently high cylinder speeds, or low enough illumination, no nystagmus is seen.

Each bird was put for observation into a thin glass cylinder, mounted on a bottom and lower part of wide mesh wire netting; the top is a plate of celluloid, cemented to the glass, with many circular holes punched in it. Adequate air circulation is essential. These cylinders fit neatly inside the striped cylinder of the apparatus producing flicker (*cf.* Crozier and Wolf, 1939–40 *b*), and are large enough to permit free movement of the bird. At an appropriate height in the glass cylinder a wood cross-bar is fastened as a perch.

Before the tests the birds are dark-adapted for at least 45 minutes. Then, with a fixed rotation speed of the striped cylinder, the light intensity is slowly increased until the characteristic response to the moving stripes can be noticed. At low critical intensities (and low levels of flash frequency F) the bird may turn completely around on its perch. At higher levels they are seen to be "restless" before arrival at the intensities critical for nystagmus. There is no difficulty, however, in recognizing the onset of the typical repeated head motions; the twisting of the neck so characteristic of many birds when viewing an object often appears, and finally the crouching and the opening of the beak. In darkness these birds are always quiet, but in contrast to frogs or horned toads (Crozier and Wolf, 1939–40 *a*, 1940–41 *a*) they are always "on the alert" as soon as the least light reaches them. The proper dark adaptation of the observer is of course essential.

III

The observations are summarized in Table I. The three measurements made on each individual are averaged to give I_1, and the mean of the four values of I_1 is given for each F. The P.E.$_1$'s are for the dispersion of I_1; they would be larger if suitably corrected for the small number of cases, but we are concerned simply with the form of the law for P.E.$_1$ as a function of I_m and with the criteria of internal homogeneity in the measurements. The curve shown in Fig. 1 is not changed if the plotted values of I_m are obtained by simply averaging the twelve readings at each F; the four birds used are essentially equivalent. The first series of measurements, at $F = 20$, gave the result bracketed in Table I and plotted as an open circlet in the figures; although I_m was a little high, it does not really depart by a significant amount from the value obtained at $F = 20$ at the conclusion of the whole series, as Fig. 2 demonstrates. The observations were arranged to show any influence of training during the repeated tests, but no effect of this sort can be detected.

The log I_m data of Table I are plotted in Fig. 1. To them has been adjusted a normal probability integral (*cf.* also Fig. 2). The description by this curve must be regarded as excellent, particularly in view of the comparatively small number of observations at each point. The flicker response contour for the finch may therefore be placed with those of *Pseudemys* (turtle), *Sphaerodactylus* (gecko), *Phrynosoma* (lizard), and *Asellus* (isopod) as example of a simplex performance curve; each of these (*cf.* Crozier, Wolf, and Zerrahn-Wolf, 1938, 1938–39 *a*, *b*; Crozier and Wolf,

1938, 1938–39, 1940–41 *a*) is also well described by the probability integral. In keeping with the simplex character of the curve in Fig. 1, the ratio of P.E.$_{1_{I_1}}$ to I_m is statistically constant (Fig. 3) over the whole range (*cf.* Crozier, Wolf, and Zerrahn-Wolf, 1938–39 *a*; Crozier and Wolf, 1939–40 *a*, etc.). The band in Fig. 3 is divided arithmetically in half on the ordinate; the equality of distribution of the points in the two halves is a test of the homogeneity of the sets of measurements (*cf.* Crozier and Holway, 1938, 1939–40; Holway and Crozier, 1937).

TABLE I

Flicker response critical flash intensities for the zebra finch (*Taeniopygia castanotis* (Gould)) at fixed flash frequencies F per second, with equality of light and dark time; log I_m (millilamberts) gives the mean intensities from three observations on each of the same four male individuals at all points. (The figures in parentheses are from an initial set of exploratory measurements.) The dispersions of the individual means are given under log P.E.$_{1_{I_1}}$; these are not corrected for the small sample size.

F	log I_m	log P.E.$_{1_{I_1}}$
2	$\bar{6}.7087$	$\bar{7}.1683$
4	$\bar{5}.3677$	$\bar{7}.7801$
6	$\bar{5}.6354$	$\bar{6}.0096$
10	$\bar{4}.1268$	$\bar{6}.5777$
15	$\bar{4}.6408$	$\bar{6}.5366$
20	($\bar{3}.3477$	$\bar{4}.3065$)
	$\bar{3}.1389$	$\bar{6}.7853$
25	$\bar{3}.6245$	$\bar{4}.0811$
	$\bar{3}.5594$	$\bar{4}.0623$
30	$\bar{3}.8919$	$\bar{4}.1854$
35	$\bar{2}.2463$	$\bar{4}.9473$
40	$\bar{2}.7037$	$\bar{3}.3724$
	$\bar{2}.6431$	$\bar{3}.1410$
45	$\bar{1}.1086$	$\bar{3}.4946$
48	$\bar{1}.5168$	$\bar{3}.8106$
50	$\bar{1}.9530$	$\bar{2}.4428$
52	0.4344	$\bar{2}.6329$
53	0.8868	$\bar{1}.5568$
54	1.2785	$\bar{1}.1034$
55	2.0302	$\bar{1}.8498$
	2.0370	$\bar{1}.6884$

IV

The smoothly symmetrical character of the F—log I_m graph in Fig. 1 indicates the absence of special effects, with $t_L = t_D$, due to movements of the iris, or to the presence of a pecten (*cf.* Menner, 1938). In this respect the curve is similar to that for *Phrynosoma* (Crozier and Wolf, 1940–41 *a*), although situated at a much lower intensity level, the abscissa of inflection τ' ($\bar{3}.73$) being 2.90 log units less. The slope is also much lower for the

finch curve, being only a little greater than that for *Pseudemys* (Crozier, Wolf, and Zerrahn-Wolf, 1938–39 *a*); by extrapolating the data for the dependence of the *Pseudemys* τ' on temperature, for flash cycles with $t_L = t_D$ (Crozier, Wolf, and Zerrahn-Wolf, 1938–39 *a*; Crozier and Wolf, 1939–40 *b*), τ' at 42°C. would be about $\bar{3}.8$. This indicates a fairly close parallelism between the cone curves for *Pseudemys* and the finch, as the values of $F_{\text{max.}}$ are also fairly close together (52.6 and 55.25), although the slope of the finch

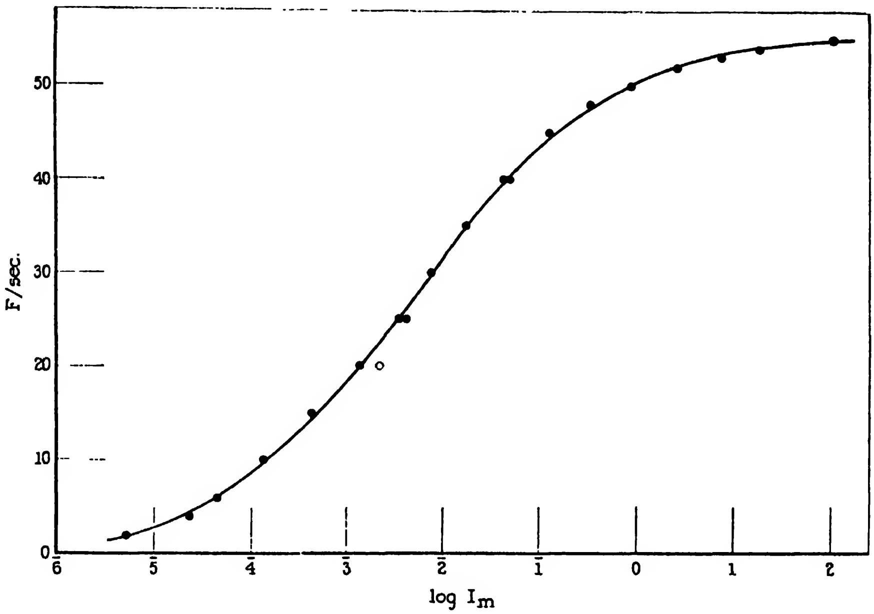

Fig. 1. The relation between flash frequency F and log mean critical flash intensity (I_m), with the light-time fraction = 0.5, for the zebra finch (*Taeniopygia castanotis* (Gould)) (cone retina). Data in Table I. The simplex curve is a normal probability integral.

curve is greater. In thus comparing the flicker acuity of the finch with that of other forms, even when correction is made for body temperature, it should be remembered, however, that we are here discussing data in which $t_L = t_D$ and the opaque moving bars cover the whole visual field. With the presence of the pecten, the sensory effects produced by a single moving image might be of quite a different order. An indication in this direction might well be given by tests in which t_L/t_D is varied systematically; certainly we have reason to know that the quantitative dependences of the two parameters of the curve sensitive to this variable, namely $F_{\text{max.}}$ and τ', are different in different animals (*cf.* Crozier and Wolf, 1939–40 *c*, 1940–41 *b*).

Under the conditions of test the simplex cone curve of the finch runs to lower critical intensities than does that for *Pseudemys*, or for man (*cf.* Crozier, Wolf, and Zerrahn-Wolf, 1937–38 *a*; Crozier and Wolf, 1940–41 *b*). A casual commentator might easily venture the thought that in general the

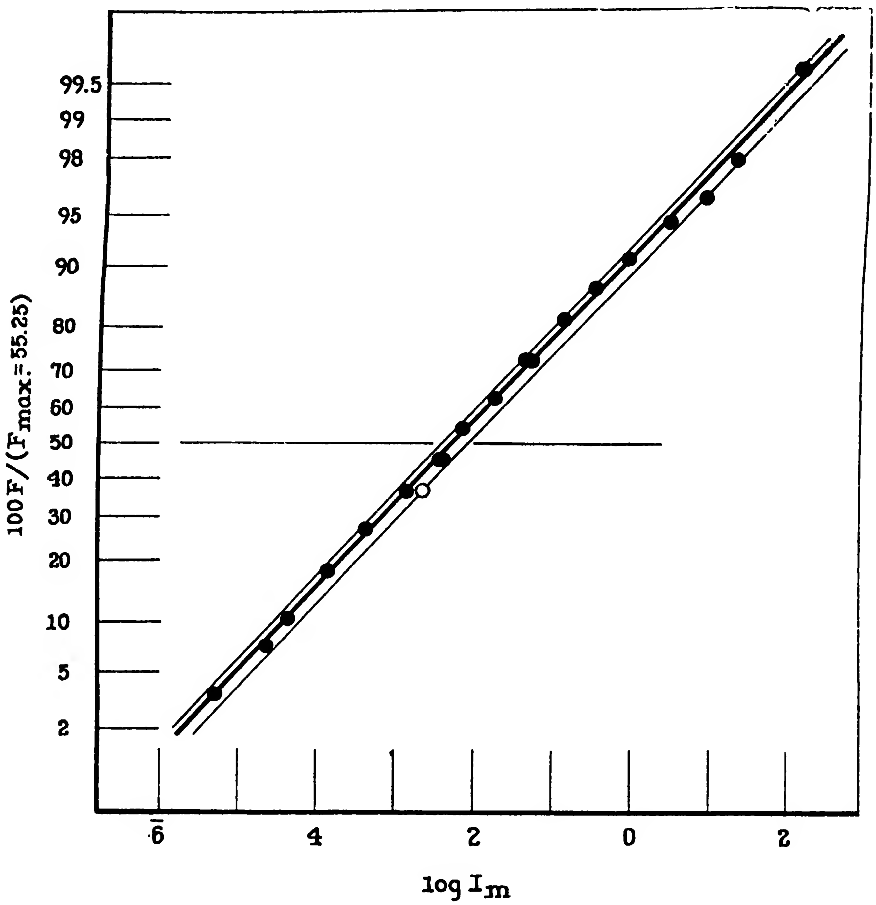

FIG. 2. The graph of Fig. 1 on a probability grid; see text

form of the F — log I curve could be influenced, or even determined, by the "visual acuity" curve of the human observer. The latter is unquestionably a duplex affair (*cf.* Hecht, 1937); consequently, having decent respect for the probability that serious observers are likely to be rationally sophisticated with reference to such a point, it is only necessary to refer to the fact that within the low intensity range concerned the observers find (*a*) specific types of duplexity in the response contours of diverse vertebrates (*cf.* Crozier, Wolf, and Zerrahn-Wolf, 1937–38; Crozier and Wolf, 1938–39,

1939–40 *a, b, d,* etc.), but also (*b*) perfectly symmetrical lower ends to the visual response contour, in *Pseudemys*, in the gecko, in *Asellus* (Crozier, Wolf, and Zerrahn-Wolf, 1938–39 *b*), and in the present data on the finch. The simplex or the duplex character of the various measured curves therefore cannot possibly be regarded as due to the visual limitations of the human observer.

The existence of simplex performance contours for certain vertebrates permits an empirical test of the proposition (Hecht, 1938) that their F − log

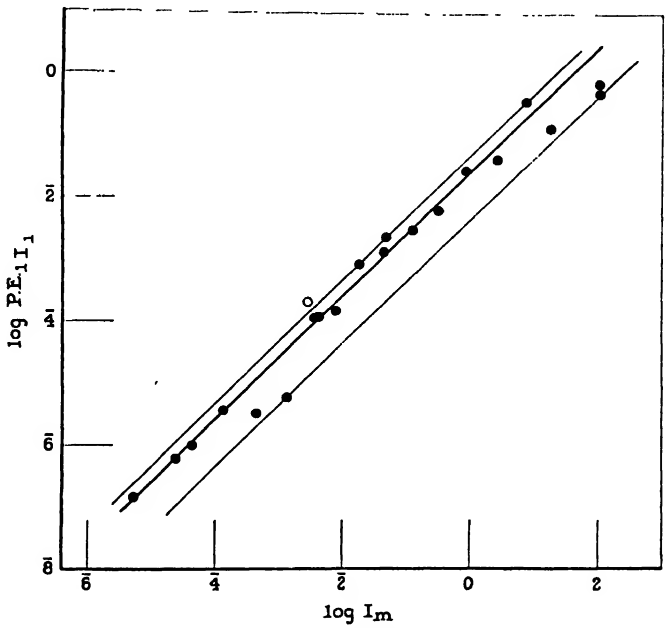

FIG. 3. The observed variation of critical flash intensity (Table I) is randomly distributed in direct proportion to I_m.

I curves can be described on the basis of photostationary state equations. We have already pointed out that, so far as concerns this situation in general, the properties of the flicker response contour are in unmistakably fundamental opposition to the idea that its shape permits deductions as to the physicochemical basis of the primary process of receptor excitation by light (*cf.* Crozier, Wolf, and Zerrahn-Wolf, 1938–39 *b*; Crozier and Wolf, 1939, 1939–40 *a*, 1940–41 *b*). It is of some formal consequence to show, however, that when the uncomplicated rod or cone curve for a vertebrate can be obtained with a known precision over an adequate range, it simply does not follow the course required by the existing photochemical theory of the properties of such data. In discussing this question we have in-

sisted on the significance of two quite different kinds of tests, namely: (1) those connected with the use of objective tests of curve fitting (with, from the classical standpoint, their inevitable limitations arising from the use of homogeneous data); these cannot be used at all, of course, unless the standard deviations of the measurements are known; and (2) those tests, theoretically much more significant, which involve experimentally determined properties of parameters in proposed descriptions.

With respect to tests of the second kind it must be said that the definite results of experiments involving the alteration of temperature, light-time fraction in the flash cycle, and other variables, are fundamentally opposed to the photochemical hypothesis (*cf.* Crozier, Wolf, and Zerrahn-Wolf, 1938–39 *a*, etc.; Crozier and Wolf, 1939, 1939–40 *a*, *c*, 1940–41 *b*). Nevertheless it is important to demonstrate that, just as for other simplex response contours obtained (Crozier, Wolf, and Zerrahn-Wolf, 1938–39 *a*), the curve for the zebra finch, while being entirely consistent with description by a probability integral (Figs. 1 and 2) simply fails to be fitted by the stationary state equation. The demonstration involves two major considerations: (1) in vertebrates to which the photochemical stationary state formulation has been applied only the upper portion of the cone curve is directly exposed for analysis; the portion of this segment which is uncomplicated by the participation of rod effects may be really very small (*cf.* Crozier and Wolf, 1939–40 *d*, etc.); and (2), the shape of the low intensity rod segment of the typically duplex curve is commonly, in man for example, the result of a complex interaction with cone effects, as suitable elementary experiments amply demonstrate (*e.g.*, Crozier and Wolf, 1940–41 *b*, etc.). Consequently, from the standpoint of mere curve-fitting, a primary significance attaches to the precise shape of the simplex contour for flicker recognition by a vertebrate, when this can be ascertained. Under the conditions of the present experiments it can be asserted that each F is effectively constant; we know that for any single measurement of I_e it is within less than 0.1 of the assigned value, while the precision with which I_m is determined is given by the fact that $\sigma_{\log I_m}$ is shown to be effectively constant. It follows that on a plot of log I_m *vs.* log F systematic departures from the stationary state equation have a significance very simple to test. The nature of the function is such that the tails are the most sensitive and significant regions.

In Hecht's (1938) scheme for such phenomena the finch data fall most nearly into line with his equation

$$Ik_1/k_2 = x^n/(a - x)^m,$$

when $n = m = 2$. For $n = m$, with any value, this equation is of course identical with the logistic $F/F_{\max.} = 1/(1 + e^{-p \log I})$ (*cf.* Crozier, Wolf, and Zerrahn-Wolf, 1936–37; and Crozier and Wolf, 1939; 1939–40 *a*).

Consequently a plot of the data on a logistic grid should at once reveal the degree to which this particular formulation is adequate. It is apparent in this plotting that, as for *Pseudemys* and *Asellus* (Crozier, Wolf, and Zerrahn-Wolf, 1938-39 *b*), the deviations are systematic and insuperably great. Therefore on this purely formal ground the photostationary state equation for the flicker response contour is to be rejected. Above $F = 0.2\ F_{max.}$ the description might not be regarded as impossibly bad, by criteria of mere inspection; this helps to explain why it has been found acceptable for the cone curves of vertebrates showing visual duplexity, since here the lower end of the curve is of course masked.

V

SUMMARY

The flicker response contour has been determined, with equality of light-dark time ratio, for the diurnal bird the Australian zebra finch. This bird has only cones in the retina. The curve of log critical intensity as a function of flash frequency is simplex, a normal probability integral. In this respect it is like that for other vertebrates not exhibiting visual duplexity. The parameters of the curve most closely approach those for the turtle *Pseudemys* (extrapolated to about the same temperature); it is not improbable that the approximation of these two curves would be less close for other values of the light-time fraction. Some points of interpretive visual theory are discussed in relation to the present measurements.

CITATIONS

Bartels, M., 1931, in Handbuch der normalen und pathologischen Physiologie, Berlin, J. Springer, **12,** 1113.

Crozier, W. J., and Holway, A. H., 1938, *Proc. Nat. Acad. Sc.*, **24,** 130. 1939-40, *J. Gen. Physiol.*, **23,** 101.

Crozier, W. J., and Wolf, E., 1938, *Proc. Nat. Acad. Sc.*, **24,** 535. 1938-39 *a*, *J. Gen. Physiol.*, **22,** 463; 1938-39 *b*, **22,** 555. 1939, *Proc. Nat. Acad. Sc.*, **25,** 171. 1939-40 *a*, *J. Gen. Physiol.*, **23,** 143; 1939-40 *b*, **23,** 229; 1939-40 *c*, **23,** 531; 1939-40 *d*, **23,** 677; 1940-41 *a*, **24,** 317; 1940-41 *b*, **24,** 635.

Crozier, W. J., Wolf, E., and Zerrahn-Wolf, G., 1936-37, *J. Gen. Physiol.*, **20,** 411; 1937-38 *a*, **21,** 17; 1937-38 *b*, **21,** 203. 1938, *Proc. Nat. Acad. Sc.*, **24,** 125. 1938-39 *a*, *J. Gen. Physiol.*, **22,** 311; 1938-39 *b*, **22,** 451.

Hecht, S., 1937, *Physiol. Rev.*, **17,** 239. 1938, *Harvey Lectures*, 1937-38, 35.

Holway, A. H., and Crozier, W. J., 1937, *Proc. Nat. Acad. Sc.*, **23,** 509.

Menner, E., 1929, *Z. vergleich. Physiol.*, **3,** 1. 1938, *Zool. Jahrb., Abt. allg. Zool. Physiol.*, **58,** 481.

Mowrer, O. H., 1936, *Psychol. Monogr.*, **47,** 294.

Rochon-Duvigneaud, A., 1919, *Ann. ocul.*, Brussels, **156,** 717.

Slonaker, J. R., 1918, *J. Morphol.*, **31,** 351.

Verrier, M.-L., 1939, *Compt. rend. Acad. sc.*, **209,** 845.

Visser, J. A., and Rademaker, G. G. J., 1934, *Arch. néerl. physiol.*, **19,** 482.

THEORY AND MEASUREMENT OF VISUAL MECHANISMS

V. Flash Duration and Critical Intensity for Response to Flicker

By W. J. CROZIER and ERNST WOLF

(*From the Biological Laboratories, Harvard University, Cambridge*)

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I

The use of the light-time fraction in the flash cycle as a variable permits a test of the nature of the visual flicker recognition contour, and helps to define the properties of the analytical elements which its investigation requires. To be really meaningful, such use depends on the determination of F-log I contours over as wide as possible a range of flash frequency and of flash intensity. The information on this subject, for the human observer, has been fragmentary and superficially somewhat confused; discussions of the photochemical hypothesis of intensive discrimination (*e.g.*, Hecht, 1937, 1938) have usually avoided reference to such data, although their general significance is as direct and fundamental as are the data on the influence of temperature (*cf.* Crozier, Wolf, and Zerrahn-Wolf, 1936–37 *b*, *c*; 1938; 1938–39; Crozier, 1939; Crozier and Wolf, 1938–39 *b*, *c*; 1939 *a*, *b*; 1940–41 *a*, *c*).

Reasonably complete F-log I curves with differing proportions of light-time (t_L) to dark-time (t_D) in the flash cycle, using demonstrably homogeneous measurements, should be significant in several different ways: (1) in establishing the non-specific direction of the shift in the general position of the curve; (2) as a test of the efficacy of the methods proposed for the analysis of the human F-log I contour (*cf.* Crozier, Wolf, and Zerrahn-Wolf, 1936–37 *a*; Crozier and Wolf, 1940–41 *c*); and thus (3) for the measurement of the dependence of the parameters of the underlying function on the percentage light-time; this cannot be done with data obtained at a single flash frequency or at one flash intensity, but requires use of the practically complete contour; and (4), for the study of the real nature of the lower branch of the human flicker curve, commonly presumed to represent quantitatively the properties of retinal rods (*cf.* Hecht, 1934, 1937). This last point turns out to have a number of especially interesting consequences; it is pursued further in the following paper (Crozier and Wolf, 1940–41 *d*).

We have used the visual discriminometer already described (Crozier

and Holway, 1938–39 *a*) for the production and control of interrupted light (*cf.* Crozier and Wolf, 1940–41 *c*). The outer of the two right-hand beams (*cf.* Crozier and Holway, 1938-39 *a*, Fig. 1) was brought to a focus by means of a suitable lens beyond the mirror *P*, in the plane of an accurately cut black metal sector-disc rotated by the gear-shaft system already described (Crozier and Wolf, 1940-41 *c*). The additional feature of the present experiments lay in the use of a series of the sectored discs giving different proportions of light-time to dark-time in the flash cycle. The disks had either six or eight open sectors, and the dimensions and the optical properties of the system were such that sharp and complete cut-off of light was secured, even with $t_L = 0.1\ (t_L + t_D)$. The series of disks provided t_L = 10, 25, 50, 75, and 90 per cent of the flash cycle time. The design of the sectors and the driving gear system was chosen to give flash frequencies up to 80 flashes per second within the range of shaft speeds favorable to precise control by our magneto-millivoltmeter system (*cf.* Crozier and Wolf, 1939–40 *b*; 1940–41 *c*). The size of the square field, centrally fixated, was the same as that used for the preceding paper (Crozier and Wolf, 1940–41 *c*); W. J. C. served as observer, and in fact the (monocular, left eye) data for $t_L = t_D$ already used form an integral part of the series of measurements here considered. For the present purpose only monocular observations were taken (left eye).

The procedure, preliminary dark adaptation, and methods of calculation are reviewed in the preceding paper. With flash frequency *F* fixed, flash intensity is slowly increased from a level well below the fusion intensity, until the observer signals recognition of flicker. The intensity critical for flicker is a more reliable end-point than that for fusion. The readings at each *F* are taken in sets of 10, in rather rapid succession. For each series of readings observations are begun after suitable preliminary dark adaptation (25 to 60 minutes, depending on the *F* level), with lowest *F* used first and higher values in succession.

II

Table I contains the data for the present discussion, with the exception of those for t_L = 50 per cent; the latter are contained in Table I of the preceding paper (Crozier and Wolf, 1940–41 *c*), and are also used in Fig. 1.

Fig. 1 shows that as the light-time fraction is decreased the curve as a whole is moved toward *lower* flash intensities, and that the maximum level to which it rises increases. This is in agreement with the effects already obtained with lower animals, and in the really comparable earlier experiments with man (*cf.* Section III). There is only slight, but systematic,

change, however, in the morphology of the lower branch of the duplex curve. It is noteworthy that certain curious minor details of the structure of the low ("rod") segment of the F-log I_m curve are persistently present, although they might well be taken at a first glance to be no more than the result of "experimental errors." They have no correlation with the use of particular filters for the control of intensity. Their homologues appear also as shown in a subsequent paper (Crozier and Wolf, 1940–41 *d*) when "monochromatic" lights are used,—in certain cases in accentuated form; as shown in our studies of monocular and binocular excitation (Crozier and Wolf, 1940–41 *c*) they are not peculiar to one observer. They receive a natural explanation in the analysis of the duplex contour given in Section IV.

It should be stated here that in securing the data of Table I, as in other experiments conducted in the present program, the sets of readings for any given contour have been taken in overlapping groups on the F coordinate, so that systematic shifts due to practice and (so far as possible) the effects of day-to-day fluctuations and changes have been guarded against. The closeness of agreement in duplicate F measurements (Table I) taken on different occasions is a guarantee of success in this respect; the only considerable differences occur in the region of the flat "shoulder" of the bipartite curve, where they are clearly to be expected. The order in which the five curves were obtained was $t_L = 0.50, 0.25, 0.75, 0.10, 0.90$.

In some careful work on the form of the $F - \log I$ curve it has been found (Hecht and Verrijp, 1933–34; Hecht, Shlaer, and Smith, 1935; Hecht and Smith, 1935–36; Ross, 1938) that at its upper end the curve bends over, so that $F - \log I$ is not monotonic. We were at first inclined to believe that this represented a normal aspect of the flicker contour (Crozier, Wolf, and Zerrahn-Wolf, 1936–37 *b*), which might explain the nature of the declining "rod" curve in visually duplex animals; its susceptibility to the presence of a retinal "surround," and to the size of the field, would not have interfered with this view,—any more than in the analogous case of intensity discrimination (*cf.* Crozier, 1940 *b*). But we have not been able to detect the existence of the bend even in an animal with purely rod retina (*cf.* Crozier, Wolf, and Zerrahn-Wolf, 1936–37 *a*; Crozier and Wolf, 1938–39 *c*, 1940–41 *c*); it is not present in any of the purely cone vertebrates we have tested, even when the curve is favorably located on the intensity scale (*cf.* Crozier, Wolf, and Zerrahn-Wolf, 1938–39; Crozier and Wolf, 1939–40 *b*, 1940–41 *b*); and a more satisfactory explanation for the declining rod curve in duplex performance contours is now available (*cf.* Crozier and Wolf, 1938–39 *a*, and Section V of the present paper). We are required to believe that the drop which has been described for the upper end is not a part of the essential

TABLE I

Mean critical intensities (as log I_m, *millilamberts*) for recognition of flicker, as a function of flash frequency F per second, with different proportions of light-time in the flash cycle; 10 observations at each point; $\text{P.E.}_{1 I_1}$ is for the dispersion of the 10. Monocular (left eye), W. J. C. White light. Field 5° square, centrally fixated.

F	t_L, *per cent*							
	10		25		75		90	
	log I_m	log $\text{P.E.}_{1 I_1}$	log I_m	log $\text{P.E.}_{1 I_1}$	log I_m	log $\text{P.E.}_{1 I_1}$	log I_m	log $\text{P.E.}_{1 I_1}$
2	$\bar{5}.3016$	$\bar{7}.8261$	$\bar{5}.6970$	$\bar{7}.9830$	$\bar{4}.3686$	$\bar{5}.7423$	$\bar{4}.6821$	$\bar{5}.2880$
	$\bar{5}.3358$	$\bar{7}.8589$			$\bar{4}.2918$	$\bar{6}.7931$		
3	$\bar{5}.4051$	$\bar{7}.6996$	$\bar{5}.7305$	$\bar{6}.1535$	$\bar{4}.4524$	$\bar{6}.8695$	$\bar{4}.7760$	$\bar{5}.0780$
4	$\bar{5}.4761$	$\bar{6}.3405$	$\bar{5}.7880$	$\bar{6}.1551$	$\bar{4}.5107$	$\bar{6}.8018$	$\bar{4}.8340$	$\bar{5}.2907$
5	$\bar{5}.5276$	$\bar{7}.8909$	$\bar{5}.8502$	$\bar{6}.3449$	$\bar{4}.5931$	$\bar{6}.9490$	$\bar{4}.9183$	$\bar{5}.3866$
6	$\bar{5}.6029$	$\bar{7}.8508$	$\bar{5}.9654$	$\bar{6}.3680$	$\bar{4}.6807$	$\bar{6}.9947$	$\bar{3}.0145$	$\bar{5}.3780$
7	$\bar{5}.7008$	$\bar{6}.0098$	$\bar{4}.0290$	$\bar{6}.5050$	$\bar{4}.7464$	$\bar{5}.1553$	$\bar{3}.0842$	$\bar{5}.4385$
8	$\bar{5}.7473$	$\bar{6}.2487$	$\bar{4}.1109$	$\bar{6}.3319$	$\bar{4}.7931$	$\bar{5}.5770$	$\bar{3}.1458$	$\bar{5}.6642$
9	$\bar{5}.8721$	$\bar{6}.0475$	$\bar{4}.2201$	$\bar{6}.6583$	$\bar{4}.9024$	$\bar{5}.2984$	$\bar{3}.2296$	$\bar{5}.6401$
10	$\bar{5}.9068$	$\bar{6}.4283$	$\bar{4}.2742$	$\bar{6}.5700$	$\bar{3}.0334$	$\bar{5}.5520$	$\bar{3}.3475$	$\bar{5}.7938$
11	$\bar{4}.0212$	$\bar{6}.5327$	$\bar{4}.3624$	$\bar{6}.9571$	$\bar{3}.0934$	$\bar{5}.3908$	$\bar{3}.4067$	$\bar{4}.1744$
12	$\bar{4}.0931$	$\bar{6}.5342$	$\bar{4}.4462$	$\bar{6}.7331$	$\bar{3}.1729$	$\bar{5}.5854$	$\bar{3}.5386$	$\bar{5}.9108$
13	$\bar{4}.1835$	$\bar{5}.1336$	$\bar{4}.5309$	$\bar{5}.1001$	$\bar{3}.2785$	$\bar{5}.7004$	$\bar{3}.6077$	$\bar{4}.0136$
14	$\bar{4}.2467$	$\bar{6}.5752$	$\bar{4}.6256$	$\bar{5}.0648$	$\bar{3}.3852$	$\bar{5}.7331$	$\bar{3}.7157$	$\bar{4}.1770$
15	$\bar{4}.3564$	$\bar{6}.7862$	$\bar{4}.7310$	$\bar{5}.0991$	$\bar{3}.4794$	$\bar{5}.9490$	$\bar{3}.7748$	$\bar{4}.2426$
16	$\bar{4}.5111$	$\bar{6}.9174$	$\bar{4}.8873$	$\bar{5}.4038$	$\bar{3}.6360$	$\bar{4}.2114$	$\bar{3}.9452$	$\bar{4}.2943$
17	$\bar{4}.6872$	$\bar{5}.1553$	$\bar{3}.0212$	$\bar{5}.2548$	$\bar{3}.7931$	$\bar{4}.1866$	$\bar{2}.1415$	$\bar{4}.5308$
18	$\bar{4}.8447$	$\bar{5}.3556$	$\bar{3}.2235$	$\bar{5}.5487$	$\bar{2}.0017$	$\bar{4}.4277$	$\bar{2}.3395$	$\bar{4}.6807$
19	$\bar{3}.0899$	$\bar{5}.4063$	$\bar{3}.4853$	$\bar{5}.8573$	$\bar{2}.2375$	$\bar{4}.7437$	$\bar{2}.5936$	$\bar{3}.0319$
			$\bar{3}.4923$	$\bar{5}.6609$			$\bar{2}.5711$	$\bar{3}.0450$
20	$\bar{3}.7679$	$\bar{4}.2963$	$\bar{2}.1139$	$\bar{4}.6774$	$\bar{1}.0748$	$\bar{3}.6284$	$\bar{1}.4128$	$\bar{3}.8168$
	$\bar{3}.4373$	$\bar{5}.8924$	$\bar{2}.1099$	$\bar{4}.5385$	$\bar{1}.0611$	$\bar{3}.4356$		
			$\bar{2}.1380$	$\bar{4}.5988$				
22	$\bar{2}.2638$	$\bar{4}.8174$	$\bar{2}.6235$	$\bar{3}.1556$	$\bar{1}.4227$	$\bar{3}.8381$	$\bar{1}.7439$	$\bar{2}.1413$
			$\bar{2}.6079$	$\bar{4}.9433$				
25	$\bar{2}.4944$	$\bar{3}.0475$	$\bar{2}.8591$	$\bar{3}.1617$	$\bar{1}.6439$	$\bar{2}.2475$	0.0120	$\bar{2}.4696$
			$\bar{2}.8754$	$\bar{3}.5540$				
28	$\bar{2}.7806$	$\bar{3}.0574$	$\bar{1}.1082$	$\bar{3}.7486$	$\bar{1}.8591$	$\bar{2}.2332$	0.1749	$\bar{2}.4604$
30	$\bar{2}.8957$	$\bar{3}.5180$	$\bar{1}.2690$	$\bar{1}.8215$	0.0103	$\bar{2}.4511$	0.3506	$\bar{2}.8742$
			$\bar{1}.2594$	$\bar{2}.0408$				
33	$\bar{1}.1242$	$\bar{3}.5914$	$\bar{1}.4589$	$\bar{3}.8132$	0.2175	$\bar{2}.5342$	0.5084	$\bar{2}.9756$
35	$\bar{1}.2801$	$\bar{3}.8132$	$\bar{1}.6150$	$\bar{2}.1374$	0.3935	$\bar{2}.7479$	0.6929	$\bar{1}.2278$
			$\bar{1}.6221$	$\bar{2}.1966$				
38	$\bar{1}.4691$	$\bar{3}.9978$	$\bar{1}.8232$	$\bar{2}.3315$	0.5804	$\bar{1}.0506$	0.9352	$\bar{1}.4693$
40	$\bar{1}.8440$	$\bar{2}.3208$	$\bar{1}.9736$	$\bar{2}.3199$	0.8004	$\bar{1}.1263$	1.1383	$\bar{1}.4541$
	$\bar{1}.6443$	$\bar{3}.8819$	0.0124	$\bar{2}.4806$				
43	$\bar{1}.9681$	$\bar{2}.4087$	0.3346	$\bar{2}.8403$	1.0322	$\bar{1}.4153$	1.3591	$\bar{1}.7661$
	0.1464	$\bar{2}.5864$						
45	0.1901	$\bar{2}.8721$	0.5475	$\bar{2}.8653$	1.2657	$\bar{1}.7359$	1.6063	0.1626
			0.5188	$\bar{1}.1028$				
48	0.5165	$\bar{1}.0327$	0.8978	$\bar{1}.5589$	1.7133	0.4631	2.0103	0.5916
49			1.0496	$\bar{1}.4153$				

TABLE I—*Concluded*

F	t_L, per cent: 10, log I_m	10, log P.E.$_{1 I_1}$	25, log I_m	25, log P.E.$_{1 I_1}$	75, log I_m	75, log P.E.$_{1 I_1}$	90, log I_m	90, log P.E.$_{1 I_1}$
50	0.9559	$\bar{1}$.6432	1.2639	$\bar{1}$.6383	2.0667	0.4814	2.3806	0.9385
			1.3345	$\bar{1}$.5530				
51			1.3273	$\bar{1}$.7581	2.2095	0.5262	2.5530	1.2829
52	1.1183	$\bar{1}$.5038	1.5167	0.2190	2.3922	1.0440	2.8766	1.4835
53					2.6460	1.1825	3.3504	1.9061
54			1.7733	0.2190	3.1159	1.6918		
			1.8642	0.5577				
55	1.6531	0.4004	1.7358	0.3206				
56			2.2322	0.6558				
57			2.7540	1.3301				
			2.7545	1.6056				
59	2.6797	1.2100						
60	3.7815	2.5973						
	3.2350	1.7118						

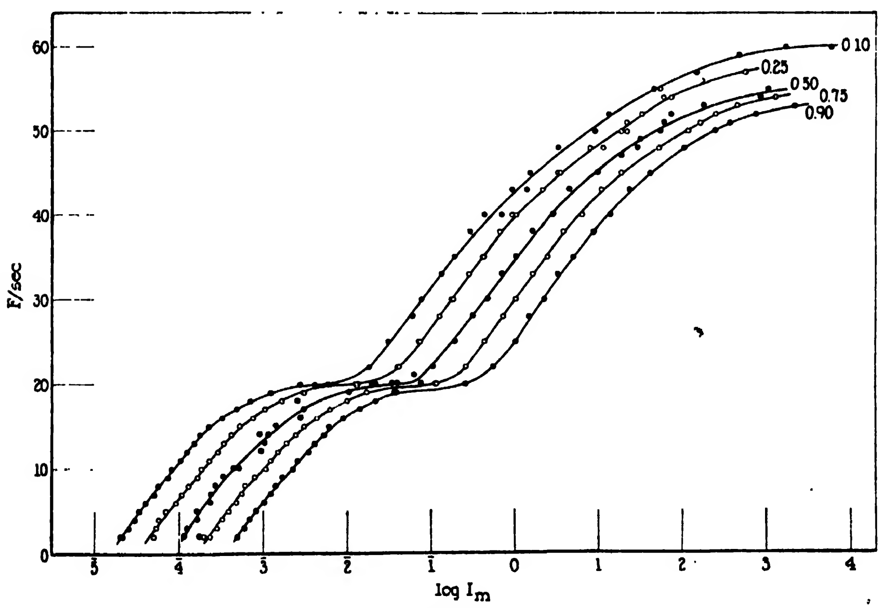

FIG. 1. Log mean critical intensity of flash as a function of flash frequency, for five values of the light-time fraction $t_L/(t_L + t_D)$. Monocular (left eye, W. J. C.), white light, 6.1° square centrally fixated; for $t_L = 0.50$, measurements from Crozier and Wolf, 1940–41 *c*; data of the other four curves from Table I. Curves drawn free-hand.

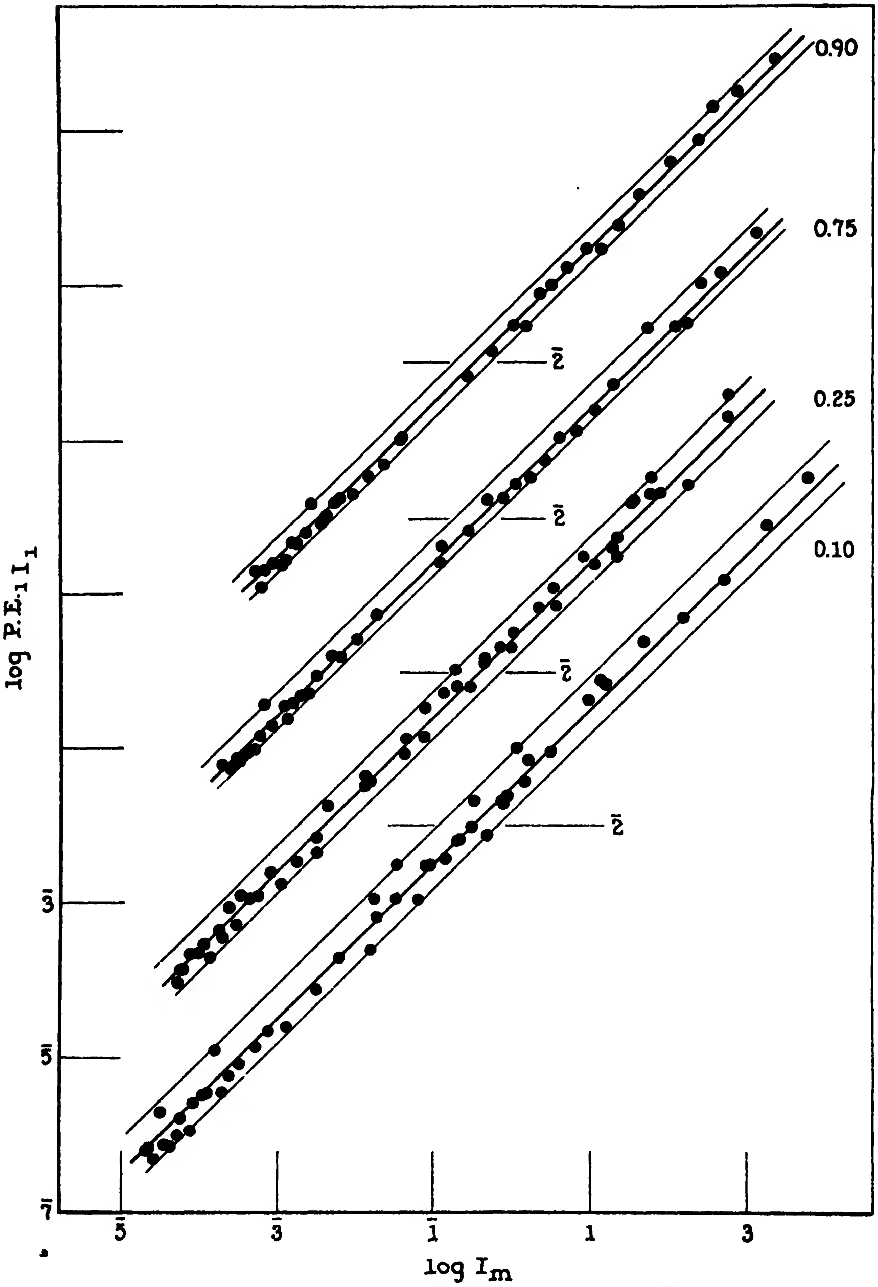

FIG. 2. I_m and $P.E._{1_{I_1}}$ are in direct, rectilinear proportion, for each value of the light-time fraction; data in Table I. For each value of the light-time fraction the intercept at log P.E.$_{1_I}$ = $\bar{2}$.00 is indicated, and is essentially the same. See text.

phenomenon, but represents either some kind of artifact or a complex effect of the use of flicker *fusion* as an end-point,—at least within the range of intensity here involved.

The readings of critical intensity were taken in sets of 10 at each F. The averages of these 10 are entered in Table I, together with the P.E.'s for the dispersions. At each value of t_L/t_D the relation between I_m and P.E.$_1$ is one of rectilinear proportionality, as proved by Fig. 2: log I_m *vs.* log P.E.$_1$ gives a band with parallel edges and a slope of 1. Using the full eye, or a sufficiently large retinal field, the characteristic form of this plot for various visually duplex animals exhibits a distinct "break" at the intensity corresponding to the complete suppression of the rod effects (*cf.* Crozier, 1935–36; Crozier, Wolf, and Zerrahn-Wolf, 1936–37 *a*; Crozier and Wolf, 1940–41 *c*). We cannot be certain of it as yet, but we may suspect that the size of field used in the present experiments, leading to a small rod group of effects, is responsible for the absence of discontinuity in the plots of Fig. 2 (*cf.* also Crozier and Wolf, 1940-41 *c*, *d*).

The absolute size of the proportionality constant (mean P.E.$_1/I_m$) is 0.0340 ± 0.005 (corresponding to an average precision of each *mean* I of *ca.* 1 per cent); this is a little less than the corresponding quantity for our determinations of ΔI (*cf.* Crozier and Holway, 1939–40), but does not differ significantly from the value already gotten from flicker experiments with the same observer using an entirely different type of apparatus and method for the adjustment of the critical intensity (Crozier, Wolf, and Zerrahn-Wolf, 1936–37 *a*); it is not really a smaller precision than that apparent in our flicker experiments with lower animals, because there the method of calculation of I_m is not the same.

III

For such sufficiently different animals as the sunfish *Enneacanthus* (Crozier, Wolf, and Zerrahn-Wolf, 1936–37 *c*), nymphs of the dragonfly *Anax* (Crozier, Wolf, and Zerrahn-Wolf, 1936–37 *b*; Crozier and Wolf, 1938–39 *d*), and the turtle *Pseudemys* (Crozier, Wolf, and Zerrahn-Wolf, 1938–39; Crozier and Wolf, 1939–40 *b*) it has already been shown that changes of the light-time fraction in the flash cycle lead to modifications of a consistent type in the $F - \log I$ contours. With increase of the t_L proportion the curve is reduced in ordinate extent and moved toward *higher* flash intensities. The fundamental shape of the curve is not changed, however. The rather widely separated rod and cone portions of the duplex contour for tne sunfish are enlarged in about the same way, quantitatively, when t_L/t_D is reduced—although these two segments are affected in different ways when the temper-

ature is changed (despite the identity of their temperature characteristics for the extent of shift; Crozier, 1939; Crozier and Wolf, 1938–39 *b*). With *Anax* the shape of the lower part of the $F - \log I$ curve does change when t_L/t_D is modified, but this is a secondary, mechanical consequence of the convexity of the optic surface (*cf.* Crozier, Wolf, and Zerrahn-Wolf, 1937–38 *c*; Crozier and Wolf, 1939 *c*).

When t_L/t_D is changed the $F - \log I$ contour alters in such a way that $F_{\text{max.}}$ decreases while the abscissa of inflection ($\log I_{\text{infl.}} = \tau'$) increases as the percentage light-time is made greater. The spread constant $\sigma'_{\log I}$, computed for $F_{\text{max.}} = 100$ per cent, does not change. These are the three parameters of the normal probability integral which describes the dependence of flash intensity upon flash frequency. Empirically, $F_{\text{max.}}$ and τ' is each a rectilinear function of $t_L/(t_L + t_D)$.

For human flicker responses the qualitative situation is the same, for both foveal and extra-foveal regions (Ives, 1922; Piéron, 1928; Cobb, 1934; Bartley, 1937; Ross, 1938), provided one is really dealing with a light-dark cycle and not with cyclic alternations of intensities of illumination (*cf.* Piéron, 1928, 1935; Crozier, Wolf, and Zerrahn-Wolf, 1937–38 *a*). For certain purposes it has been the practice (Piéron, 1928; Cobb, 1934; Ross, 1938) to deal with the relations between fusion frequency and the light-time fraction at "constant brightness." Objection to this procedure, which involves compensating for the light-time fraction by increasing the flash intensity on the basis of the Reciprocity law, is that it assumes that this rule really applies *at* the critical fusion frequency; in fact it does not. When the data are considered simply in terms of flash intensity, without this "adjustment," their analysis presents no difficulty (Crozier, Wolf, and Zerrahn-Wolf, 1937–38 *a*, *b*; Crozier and Wolf, 1940–41 *c*).

The theory proposed for the explanation of the dependence of the $F - \log I_m$ contour on the light-time fraction (Crozier, Wolf, and Zerrahn-Wolf, 1937–38 *a*) considers that in a given population of potentially excitable neural elements, giving a frequency distribution of $dF/d \log I$, the size of the population is enlarged by increase of the percentage dark-time. On this view the maximum value of F should be a direct, rectilinear function of the percentage dark-time; and also, the abscissa of inflection of the $F - \log I$ curve should decline in the same way. These phenomena are observed (Crozier, Wolf, and Zerrahn-Wolf, 1937–38 *a*, *b*; Crozier and Wolf, 1940–41 *c*). In relation to the probability integral form of the $F - \log I$ curve the reason for these findings may be visualized in terms of the proportionately greater chance of any given flash finding a particular element potentially excitable when the dark intervals are longer. It is to be kept in

mind that the "elements" of this discussion are defined in terms of $dF/d\log I_m$ along the contour for recognition of flicker. They have been pictured as *frequencies* of nerve impulses (Crozier, 1939). If the basic excitabilities of the neural units producing these elements of excitability form a homogeneous population, the $F-\log I$ curve should not suffer change in $F_{max.}$ or in shape when the temperature is altered, but τ' should exhibit as a function of temperature the properties of the mechanism governing excitability; these phenomena are also found (Crozier, 1939; Crozier and Wolf, 1940–41 *c*; and earlier papers). It likewise follows from the statistical conception of the nature of the $F-\log I$ curve that when t_L/t_D is changed the shape constant $\tau'_{\log I}$ with $F_{max.} = 100$ should be independent of t_L/t_D, as found (Crozier, Wolf, and Zerrahn-Wolf, 1937–38 *a*, *b*; Crozier and Wolf, 1939–40 *b*).

It will be shown presently that the rule obtains for the data on man, with a complication in the rod segment of the curve due to the peculiar and significant interplay of rod and cone effects. Since the type of law is thus the same for dragonfly nymph, sunfish, turtle, and man the foundation for its generality must be presumed due to a common feature of organization, namely the statistical character of the production of sensory effects responsible for the intensive discrimination involved in the recognition of flicker.

The increasing efficiency of briefer flashes (at constant cycle time) in forcing the reaction of flicker recognition cannot be quantitated by considering the flash intensities (or the equivalent brilliances) at any fixed F, because (Crozier, Wolf, and Zerrahn-Wolf, 1937–38 *a*, *b*) this function depends on the level of F selected. It is necessary to have the full range of $F-\log I$ for various values of t_L/t_D before interpretation is possible; our present human data run much more nearly over the explorable range of t_L/t_D and of intensity than have those hitherto available. It should be pointed out, however, that the increased effectiveness of a given intensity with prolongation of dark-time (*cf.* Cords, 1908) can be pushed to a considerably greater value by employing electrical excitation of the retina, and thus using values of the duration of excitation smaller than the $t_L/t_D = 1/9$ to which we are limited in the present experiments; fusion frequencies as high as $f = 98$ to 172 have been obtained (Cords, 1908; Bouman, 1935). A dynamical parallel is of course provided by the increase of photosynthetic efficiency of flashing light when the light-dark ratio is decreased (*cf.* Warburg, 1928; Emerson and Arnold, 1931–32; Arnold, 1935; Pratt and Trelease, 1938).

IV

The analysis of the curves in Fig. 1 is made in the way followed in earlier communications (*cf.* Crozier and Wolf, 1940–41 *c*). The high intensity segment of each of the curves in Fig. 1 is described by a normal probability integral. These, together with the curve for $t_L/t_D = 1$, are shown in Fig. 3. Their extrapolations toward $F = 0$ (Fig. 8) give the basis for the dissection of the rod contributions to the lower segments of the curves in Fig. 1.

Recognizing that there is a variable uncertainty in the adjustment of the lines on the probability grid, and that there is apparently a real temporal fluctuation in the parameters of the function, it is fair to conclude that the slopes of the five lines in Fig. 3 do not differ significantly. Certainly they

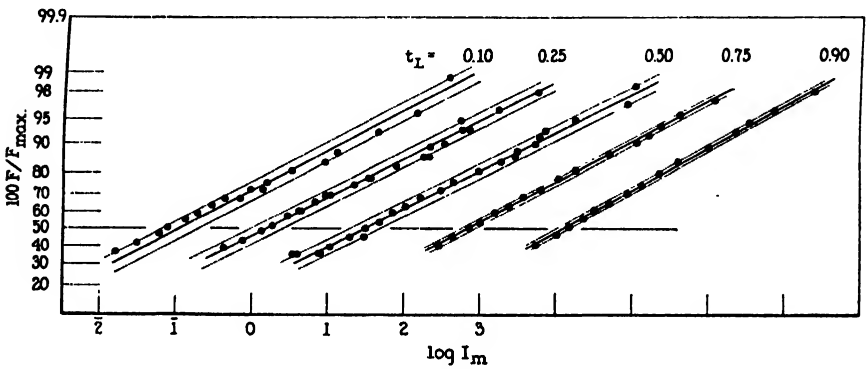

FIG. 3. The upper segments of the data in Fig. 1 put on a normal probability grid; the five lines displaced laterally for clearness. See text.

do not change systematically as a function of t_L/t_D. It can be pointed out that the subsequently determined properties of the several rod curves (Section V) supply a significant check upon the propriety of the lines drawn in Fig. 3. Hence the parameter $\sigma'_{\log I}$ is judged to be independent of the percentage light-time.

Fig. 2 demonstrates that from the standpoint of the relation between I_m and P.E.$_{1I_1}$ the measurements of each series are not of course homogeneous in the sense that the band on the log—log plot is divisible *arithmetically* into two zones with equal numbers of points (*cf.* Holway and Crozier, 1937; Crozier and Holway, 1938, 1939–40); since sets of readings were necessarily taken at different sittings, the population of P.E.'s is not homogeneous; hence a division of the log width of the scatter band into half gives equally populated zones. The extent of the relative scatter of the variation index at any level of I_m is a function of t_L/t_D, but its *mean* value is not—as is readily seen in Fig. 2. In correlation with the increased scatter

of P.E.$_{.1I_1}$ as the light-time percentage decreases, we note that in Fig. 3 the log I_m-width of the scatter band decreases steadily as we pass from t_L = 0.1 to 0.9 (Table II). We note also that this is related to the fact that the total size of the populations of excitable elements ($\equiv F_{max.}$) declines in just the same way, so that within the limits of precision of the measurements the scatter ratio for P.E.$_{.1I_1}$ (arithmetic) is directly proportional to $F_{max.}$. A better way of showing this ($F_{max.}$ and $t_L/(t_L + t_D)$ being

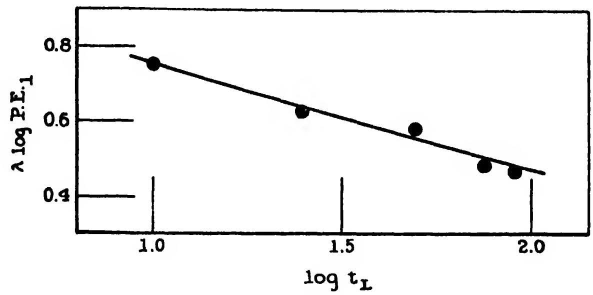

FIG. 4. The ordinate breadth λ of the scatter band for log P.E.$_{.1I}$ (from Fig. 2) is a declining rectilinear function of log (per cent light-time).

TABLE II

The values of the asymptotic $F_{max.}$ taken for the curves in Fig. 3; the corresponding values of the abscissa of inflection (τ'), for different light-time percentages; and, from Fig. 2, the ordinate height of the scatter-band for P.E.$_{.1}$ on the log scale. See text.

t_L	$F_{max.}$	τ'	λ log P.E.$_{.1}$
per cent	*per sec.*		
10	59.8	$\bar{2}.98$	0.75
25	58.3	$\bar{1}.18$	0.63
50	56.0	$\bar{1}.49$	0.58
75	55.65	$\bar{1}.85$	0.48
90	54.2	0.12	0.47

in direct proportion—Fig. 7) is to take the vertical breadth λ log P.E.$_{.1}$ of the bands in Fig. 2 as a function of the fixed values of the percentage light-time. Since, as Fig. 4 shows, λ log P.E.$_{.1}$ is rectilinearly related to log $[t_L/(t_L + t_D)]$, the ratio of the upper to the lower limit of relative scatter of P.E.$_{.1}$ changes at the same proportionate rate as does the percentage light-time.

This same type of direct correlation between the latitude of scatter (Fig. 3) and the size of the total population of potentially excitable elements is systematically exhibited in other connections,—for example, in the measurements of dark adaptation (Crozier, 1940 *a*); in a slightly different way it is shown by the comparison of monocular and binocular data on

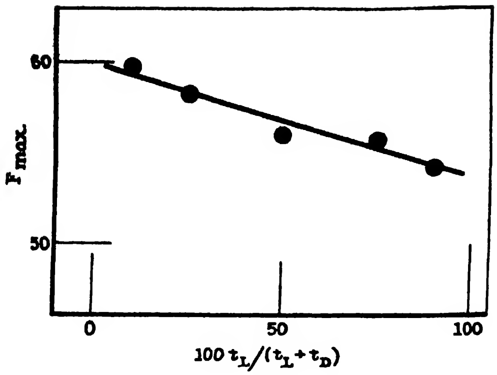

FIG. 5. $F_{max.}$ as a function of percentage light-time. (The departures from rectilinearity are not significant.)

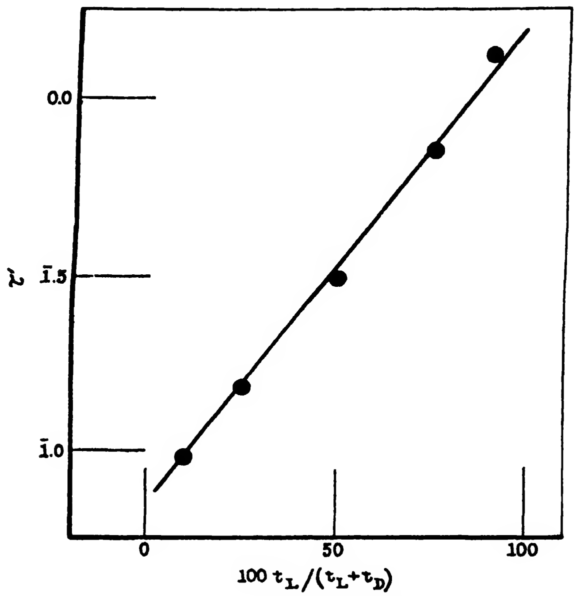

FIG. 6. The abscissa of inflection (τ') of the high intensity F-log I_m curve is directly proportional to the percentage light-time.

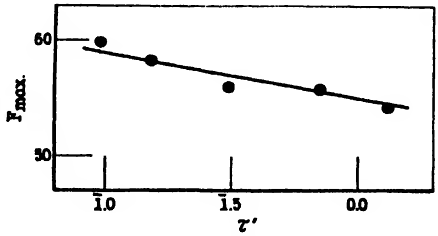

FIG. 7. The relation between $F_{max.}$ and the abscissa of the cone inflection τ' (*cf.* Figs. 5 and 6), for different light-time fractions.

intensity discrimination (Crozier and Holway, 1939–40) and on flicker (Crozier and Wolf, 1940–41 *c*). It has an important connection with the doctrine that the capacity to vary discriminative performance is organically determinate (Crozier, 1936; 1935–36) and governs the magnitude of the intensity threshold for discrimination.

We have already indicated that the asymptotic level of $F_{max.}$ and the abscissa of inflection τ' are expected (Table II) to be (reversed) rectilinear functions of the percentage light-time, and therefore themselves in direct proportion. Figs. 5, 6, and 7 show that these expectations are satisfactorily met in the data. The dependence of $F_{max.}$, and of τ', on the light-time percentage differs quantitatively in the several animal forms examined; for $F_{max.}$ *vs.* t_L the proportionality constant is greater in the present observations than with *Anax* or turtle, much less than with the sunfish; as regards τ', it is less than for any of these, all tested with white light (but is found to be even much lower when colored light is used—Crozier and Wolf, 1940–41 *d*).

V

The uncovering of the putative rod component of the duplex $F-\log I$ curves (Fig. 1) has been carried out by the methods earlier described (Crozier, Wolf, and Zerrahn-Wolf, 1936–37 *a*; Crozier and Wolf, 1940–41 *c*), already used for various other vertebrates (Crozier, Wolf, and Zerrahn-Wolf, 1937–38 *a*; Crozier and Wolf, 1938–39 *a*, 1940–41 *d*, etc.). The slight irregularities in the form of the curves in the rod region are individual and persistent; they are also found in our work with colored lights; the raw data do not conform to any simple function (*e.g.*, Hecht, 1937); when a simplex visual population of neural effects can be examined it provides a simple normal probability integral over the whole range (Crozier, Wolf, and Zerrahn-Wolf, 1938–39; Crozier and Wolf, 1938–39 *c*, 1940–41 *a*, *b*); the use of monochromatic beams in a flash cycle with large light-time ratio enables us to be sure that in the lower rod of the $F-\log I$ curve the occurrence of subjective color must by classical criteria be presumed to imply the presence of cone effects. For these reasons the cone probability integrals have been extrapolated toward $F = 0$, and the rod contributions then obtained by subtraction on the ordinates. With some lower vertebrates the separation of rod and cone branches on the log I axis is so great that the discontinuity due to the entrance of cone effects is plainly apparent on the $F-\log I$ graph (Crozier, Wolf, and Zerrahn-Wolf, 1937–38 *a*). With man, frog (Crozier and Wolf, 1939–40 *a*), and newt (Crozier and Wolf, 1939–40 *b*) the overlapping of the two cumulative populations is more extreme; the

analysis by this method, however, produces rod curves of the expected normal form. Their properties have a particular importance for the criticism of the by now more or less traditional interpretation of the relationships between rod and cone effects; they have also an immediate significance for the statistical conception of the origin of the contours of visual performance. It is to be noted that, at a given value of $t_L/t_D(=1)$, the low value of I at $F = 2$ obtained by the cone extrapolation (Fig. 8) is of the same order of magnitude as that observed with forms such as a bird (Crozier and Wolf, 1940–41 *b*) and a turtle (Crozier, Wolf, and Zerrahn-Wolf, 1938–39) which have exclusively cone retinas.

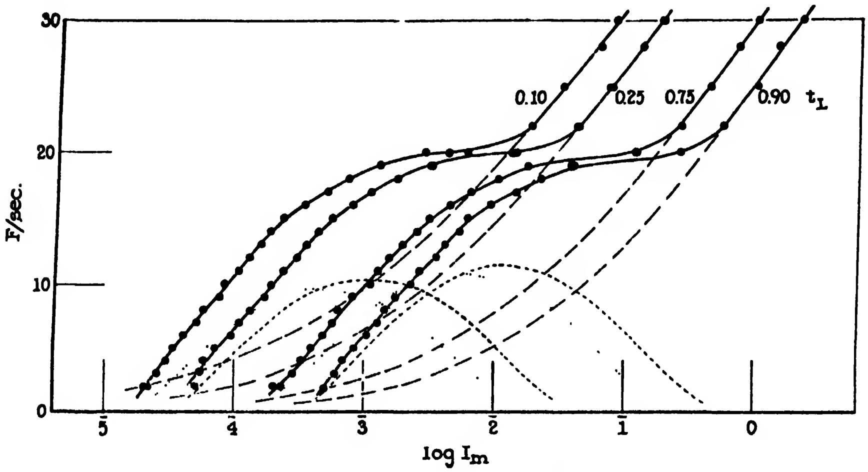

FIG. 8. The probability integrals of Fig. 3 are extrapolated toward $F = 0$, and the form of the rod contribution to the F-log I contour is then obtained by difference; see text.

The extrapolations of the cone curves and the curves obtained by differences between these and the lines put through the lower ranges of the data are shown in Fig. 8. The results for $t_L/t_D = 1$ as used in this discussion are taken from the preceding paper (Crozier and Wolf, 1940–41 *c*). The difference curves, as for all such cases, comprise an ascending and a descending branch. We originally believed (Crozier, Wolf, and Zerrahn-Wolf, 1936–37 *c*) that the declining branch was to be taken as similar in nature to the declining upper end of the cone curve as obtained by Hecht and Verrijp (1933–34), Hecht and Smith (1935–36), and Ross (1938). We are now of the view that this cone decline is a complex artifact (*cf.* Section II), however, and later evidence has led us to the opinion that the form of the declining curve obtained for the rod contributions must be understood as

the result of the progressive inhibition of rod effects as more and more cone elements are implicated (Crozier and Wolf, 1938–39 *a*). The present data seem to be uniquely consistent with that position.

Consider the form and the positions of the ascending rod curves (Fig. 8). These lines have been transferred to a probability grid and are shown in Fig. 9. For this purpose the coordinates of points on the ascending dotted curves of Fig. 8 have been read off and plotted on a large scale grid, reproduced in Fig. 9. The values of F have been computed to the maxima indicated in Fig. 8. It is apparent that the slopes of the five rectilinear

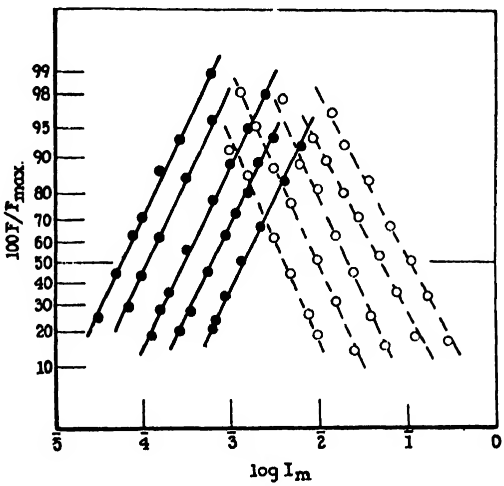

FIG. 9. The ascending and the descending branches of the (computed) rod curves of Fig. 8, points read from these curves being here transferred to a probability grid. The central curves, for $t_L = 0.50$, are taken from the preceding paper (Crozier and Wolf, 1940–41 *c*).

dispositions on the grid of Fig. 9 cannot be asserted to be different. The values of $F_{max.}$ are directly proportional to the percentage light-time (Fig. 10), but they *increase* (not decrease, as with the cone curves, Fig. 5) as the light-time fraction increases. On the other hand (Fig. 9), the slope of the *declining* rod curve decreases as t_L/t_D is made greater. The abscissa of inflection of the rod curve declines with decrease of t_L/t_D (Fig. 11), but not with the same proportionality factor as for the cone curves; for the rods this factor is smaller. With *Enneacanthus* (sunfish) we found that the non-overlapping rod and cone populations showed the same degree of dependence of τ' on t_L/t_D (Crozier, Wolf, and Zerrahn-Wolf, 1937–38 *b*), but there is no reason to suppose that this condition need prevail generally. The meaning of Fig. 9 is that the human basic rod population of effects is

under the present conditions less responsive to changes in t_L/t_D than the cone, and that the measured contribution which it makes is at each value of the light-time fraction the result of its intrinsic properties *plus* the effects of inhibition by the population of cone effects which it overlaps on the intensity scale. The declining branch of the rod curve is of decreasing slope as t_L/t_D increases for precisely the same reason that the rod $F_{max.}$ is found to increase, namely that the fundamental rod population, particularly its τ', is less sensitive to the t_L/t_D ratio and thus the inflection points for the

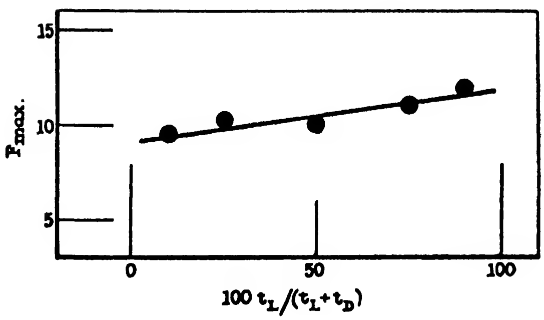

FIG. 10

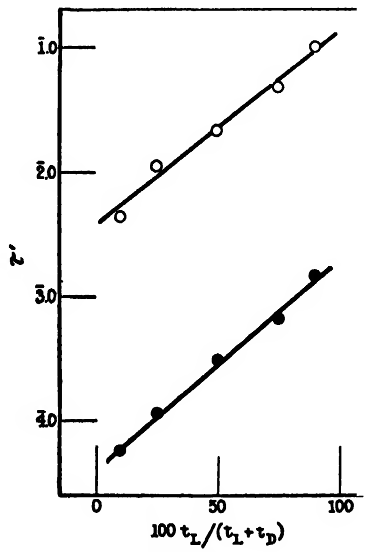

FIG. 11

FIG. 10. $F_{max.}$ for the (computed) rod curves of Figs. 8 and 9, as a function of percentage light-time.

FIG. 11. τ' for the (computed) rod curves given in Figs. 8 and 9, as a function of percentage light-time; below, for the ascending curves; above, for the descending curves.

two populations are moved farther apart by increase of t_L/t_D, and the inhibition is less effective.

The actual magnitudes of the rod $F_{max.}$ are thus to be regarded as the outcome of the actions of several distinct factors: (*a*) the separation between rod and cone abscissae of inflection is not great enough to prevent the cone population from completely underlying the rod group of effects at all points, in view of the low slope of the cone curve; (*b*) the cone τ' is more sensitive to the effect of the light-time fraction than that for the rods; and (*c*) the cone effects can partially inhibit the rod contribution.

The fact that the rising rod curves have the same $\sigma'_{\log I}$ is merely a sign

that the same population of units is engaged in the production of elements of effect despite the inhibitory action of the other group of elements, while the progressively slower decay of the rod contribution as t_L/t_D is increased, beyond the maximum, is a consequence of the relatively greater movement of the cone population toward higher intensities,—the kind of result already detected in our cross-bred teleosts (Crozier and Wolf, 1938–39 *a*). It is noted that the declining branch of the rod curve also shows a τ' rectilinearly proportional to the light-time fraction. By comparison with the results of our earlier experiment (Crozier, Wolf, and Zerrahn-Wolf, 1936–37 *a*) using a larger test area (t_L = 0.50) with the same observer, $\sigma'_{\log I}$ for both the ascending and the descending rod curves is here found to be less, although the $F_{max.}$ is about the same. This result is clearly not inconsistent with the notion of competitive relations between the cone and rod elements with respect to flicker recognition, since in the conditions of the earlier experiment the rod τ' was much smaller.

We conceive that it is difficult to construct a scheme of interpretation for these properties of the rod contribution to the duplex flicker contour other than that we have proposed. Its basic general feature is the conception of moment-to-moment fluctuation in the effective capacity of neural units involved to contribute elements of effect to the determination of the end-point response. When these effective contributions are reduced by the competitive action of another group of elements the essential parameter of the population, namely the standard deviation of its frequency distribution, is still invariant—just as when temperature or the light-time ratio is altered. With another organism, or with a test area differently located on the retina, or through the use of lights with different wave-length compositions, further aspects of these relations can be explored.

The question naturally arises as to whether the general type of "interaction" between cone and rod effects revealed by the present analysis can be detected in the data for other sorts of visual performance. The recognition of flicker is but one of various possible indices of intensive discrimination (Crozier, 1935–36), but certainly it is not necessary to suppose that the same types of interaction should appear in all. The question cannot be fully discussed without possession of a wider range of homogeneous data with respect, for example, to the rôle of retinal area. Indications already exist, however, that neural integrations of the sort appealed to are involved in the data of ordinary intensity discrimination (*cf.* Crozier and Holway, 1938–39 *b*, 1939–40), for example.

A clue as to why in the case of flicker discrimination the interactive influence is irreciprocal—cone effects inhibiting rod effects but not the reverse

—may well be given by the subjective character of the flicker end-points. On the *fusion* side of the $F-\log I$ contour, as one ascends the F scale, the following order of impressions is obtained: at low flash intensities the field is a smooth, faintly bluish gray; at higher intensities it assumes a "speckled" appearance, with few or many tiny points of yellowish light; the field then becomes "granular," the speckling dots becoming more numerous and at still higher intensities running in groups which produce a "frosted" appearance; with further increase of F the field becomes smooth. It happens that with the most commonly used conditions for flicker, namely with $t_L = t_D$, and at least with central retinal fields of moderate size (white light), the transition to the smooth whitish impression at critical fusion coincides pretty well with the kink in the $F - \log I$ curve (*i.e.*, at *ca.* $F = 20$ in Fig. 1), and that at about this level there also occurs transition from a *flicker* end-point mainly in the periphery of the illuminated field to one "finer" and definitely at the fixation center. Similar conditions have been observed with "monochromatic" fields, and have been said to be correlated with the occurrence of the *color* threshold near or just below the vicinity of the bend in the $F - \log I$ curve (*cf.* Hecht and Shlaer, 1935–36).

It would be easy to point to these correlations as indicating a sharp separation of rod and cone functions on the two branches of the curve. But the introduction of the light-time fraction as a variable shows that this is impossible. Our detailed records of the appearance of the end-point fields show that in the data of Fig. 1 the level of clear occurrence of the foveal type of end-point is at $F = 35$ for $t_L = 0.10$, 21 for 0.25, 20 for 0.50, 19 for 0.75, 16 for 0.90. When the respective flash intensities are considered, the obvious correlation is with the *mean* intensity flux, which for these values of F ranges only from anti-log $\bar{3}.90$ to $\bar{2}.18$, although apparently drifting slightly as t_L increases. This shows that no real correspondence exists between the position of the "break" in the $F - \log I$ contour and the indications of other criteria concerning the differentiation of rod and cone effects. Foveal end-points can appear ($t_L = 0.90$) a log unit and one half below the bend, or ($t_L = 0.10$) as much as a log unit above it. When colored fields are used (Crozier and Wolf, 1940–41 *c*) the discrepancies are even more impressive.

But these subjective data are thoroughly consistent with the view that a real competitive integration of rod and cone effects occurs in the recognition of flicker, and that the focal attention of the observer determines that the effects of foveal vision predominate. This is consistent with the conception of a central, rather than of a peripheral, location of the forces governing the discrimination. A number of other and quite different considerations point

to the same conclusion (*cf.* Bartley, 1938). It implies no necessity that other modes of intensive discrimination will necessarily follow exactly the same rule of competitive inhibition.

VI

SUMMARY

The relation between flash duration and mean critical intensity (white light) for threshold recognition of visual flicker, as a function of flash frequency, was investigated by means of measurements at five values of the light-time fraction: 0.10, 0.25, 0.50, 0.75, 0.90, with flash frequencies of the interrupted beam ranging from 2 to 60 per second. A square area, 6.1 × 6.1°, centrally fixated, was viewed monocularly; the discriminometer used provides automatically an artificial pupil 1.8 mm. in diameter. Except for the slight day-to-day fluctuation in the magnitudes of the parameters, the data for the observer used are shown to form an essentially homogeneous group.

As for other animals tested, the $F - \log I_m$ curve is enlarged and moved toward *lower* flash intensities as the light-time fraction is *decreased*. The high intensity segments of the duplex curves are fitted by normal probability integrals for which $F_{max.}$ and the abscissa of inflection are rectilinear functions of $t_L/(t_L + t_D)$, with opposite slopes. The third parameter, $\sigma'_{\log I}$, is invariant.

The low intensity segments are composites, their shapes determined by the summation of the lower part of the high intensity curve with an overlapping low intensity population of effects. Both the rising and the declining branches of this latter assemblage suffer competitive partial suppression by the effects in the high intensity population. The detailed analysis shows that these results are consistent with the theory of the central, rather than peripheral, location of the dynamically recognizable elements in the determination of flicker.

CITATIONS

Arnold, W., 1935, in Cold Spring Harbor symposia on quantitative biology, Cold Spring Harbor, Long Island Biological Association, **3**, 124.

Bartley, S. H., 1937, *J. Exp. Psychol.*, **21**, 678; 1938, **22**, 388.

Bouman, H. D., 1935, *Arch. néerl. physiol.*, **20**, 430.

Cobb, P. W., 1934, *J. Opt. Soc. America*, **24**, 107.

Cords, H., 1908, *Arch. Ophth.*, Leipsic, **67**, 149.

Crozier, W. J., 1935–36, *J. Gen. Physiol.*, **19**, 503. 1936, *Proc. Nat. Acad. Sc.*, **22**, 412; 1939, **25**, 78; 1940 *a*, **26**, 334; 1940 *b*, **26**, 382.

Crozier, W. J., and Holway, A. H., 1938, *Proc. Nat. Acad. Sc.*, **24**, 130. 1938–39 *a*, *J. Gen. Physiol.*, **22**, 341; 1938–39 *b*, **22**, 351; 1939–40, **23**, 101.

Crozier, W. J., and Wolf, E., 1938–39 *a*, *J. Gen. Physiol.*, **22**, 463; 1938–39 *b*, **22**, 487; 1938–39 *c*, **22**, 555; 1938–39 *d*, **22**, 795. 1939 *a*, *Proc. Nat. Acad. Sc.*, **25**, 171; 1939 *b*, **25**, 176. 1939 *c*, *Biol. Bull.*, **77**, 126. 1939–40 *a*, *J. Gen. Physiol.*, **23**, 229; 1939–40 *b*, **23**, 531; 1939–40 *c*, **23**, 667. 1940, *Proc. Nat. Acad. Sc.*, **26**, 60. 1940–41 *a*, *J. Gen. Physiol.*, **24**, 317; 1940–41 *b*, **24**, 505; 1940–41 *c*, **24**, in press; 1940–41 *d*, **24**, in press.

Crozier, W. J., Wolf, E., and Zerrahn-Wolf, G., 1936–37 *a*, *J. Gen. Physiol.*, **20**, 203; 1936–37 *b*, **20**, 393; 1936–37 *c*, **20**, 411; 1937–38 *a*, **21**, 17; 1937–38 *b*, **21**, 313; 1937–38 *c*, **21**, 463. 1938, *Proc. Nat. Acad. Sc.*, **24**, 216. 1938–39, *J. Gen. Physiol.*, **22**, 311.

Emerson, R., and Arnold, W., 1931–32, *J. Gen. Physiol.*, **15**, 391.

Hecht, S., 1934, in Murchison, C., Handbook of general experimental psychology, Worcester, Clark University Press, 704. 1937, *Physiol. Rev.*, **17**, 239. 1938, *J. Appl. Physics*, **9**, 156.

Hecht, S., and Shlaer, S., 1935–36, *J. Gen. Physiol.*, **19**, 965.

Hecht, S., Shlaer, S., and Smith, E. L., 1935, in Cold Spring Harbor symposia on quantitative biology, Cold Spring Harbor, Long Island Biological Association, **3**, 237.

Hecht, S., and Smith, E. L., 1935–36, *J. Gen. Physiol.*, **19**, 979.

Hecht, S., and Verrijp, C. D., 1933–34, *J. Gen. Physiol.*, **17**, 251.

Holway, A. H., and Crozier, W. J., 1937, *Proc. Nat. Acad. Sc.*, **23**, 509.

Ives, H. E., 1922, *J. Opt. Soc. America*, **6**, 254.

Piéron, H., 1928, *Ann. Psychol.*, **28**, 98; 1935, **35**, 1.

Pratt, R., and Trelease, S. F., 1938, *Am. J. Bot.*, **25**, 133.

Ross, R. T., 1938, *J. Gen. Psychol.*, **18**, 111.

Warburg, O., 1928, Über die katalytische Wirkung der lebenden Substanz, Berlin, J. Springer.

STUDIES IN THE PHYSIOLOGY OF FUSARIA. THE RESPIRATORY AND FERMENTATIVE MECHANISMS

By BERNARD S. GOULD AND ALFRED A. TYTELL

(*From the Laboratories of Physiology and Biochemistry, Massachusetts Institute of Technology,* Cambridge*)

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INTRODUCTION

Interest in the physiology of the *Fusaria* centers mainly about their ability to produce alcohol and carbon dioxide as practically the only end products of metabolism from a variety of carbon compounds. Early work by Wollenweber (1913) and Sherbakoff (1915) dealt mainly with the morphological differentiation of the organisms. Biochemical studies of this large group of organisms have been carried out by White and Willaman (1928) and by Birkinshaw *et al.* (1931). In the latter work detailed carbon balance sheets of the metabolism of a number of species of the organisms on a *glucose* "synthetic" medium were carried out and it was concluded that the dissimilation of glucose by these organisms led almost exclusively to the production of ethyl alcohol and carbon dioxide. In some cases, a small amount of acid was also produced. The oxidative mechanisms of these organisms and the possible presence of phosphorylating mechanisms in carbohydrate dissimilation have been investigated by Nord (1939). The results of most of the experimentation point to a possible similarity between the alcohol and carbon dioxide-producing mechanisms of *Fusaria* and those found in yeasts. Nord (1939) is of the opinion, however, that the dissimilation of glucose by *Fusaria* need not go by way of phosphorylation. This would appear to offer a point of difference between the metabolism of yeast and *Fusaria*, since MacFarlane (1939) has shown that phosphorylation does occur in living yeast cells. No extensive study has, however, been made of the various mechanisms involved in the dissimilation of glucose by *Fusaria*, especially in comparison with analogous mechanisms in glucolysis by living yeast cells. The present investigation has been planned with a view to such a comparison.

In the present investigation a study has been made of the respiratory

* Contribution No. 184 from the Department of Biology and Public Health, Massachusetts Institute of Technology.

and fermentative mechanisms of resting cell preparations of *Fusaria*. The availability of a number of carbohydrates and of certain other common compounds for carbon dioxide production by *Fusaria* was investigated, and the presence of a glucose-dissimilating mechanism which is always present and therefore "constitutive" in the sense of Karström (1938) was established, as well as the presence of an "adaptive" mechanism for the dissimilation of galactose, in a sense, similar to galactozymase in yeast (Stephenson and Yudkin, 1936). The effects on *Fusaria* metabolism of selective poisons such as iodoacetate, fluoride, and cyanide have been investigated and striking similarities to the effects on yeasts established. The possibility of more than a single glucolyzing mechanism being involved was explored, as well as the question of the presence of a glycogenetic-like process which may precede glycolysis.

Methods

Description of the Organism

The organism selected for the study is catalogued as *Fusarium* sp. *H* in the M. I. T. pure culture collection. A study of its characteristics indicates that it is closely related to *F. tricothecoides*. It was selected because of the uniformity of growth and biochemical properties.

Uniform preparations of the *Fusarium*, "free" from metabolites, analogous to "resting cell" preparations of bacteria and yeast were prepared. The organism was grown on a modified Czapek-Dox medium of the following composition: glucose, 10 gm.; peptone, 2.5 gm.; $NaNO_3$, 2 gm.; KH_2PO_4, 1 gm.; $MgSO_4 \cdot 7H_2O$, 0.5 gm.; KCl, 0.5 gm.; distilled water, 1000 ml. Into 1 liter conical flasks were introduced 100 ml. portions of the medium and these were then sterilized by autoclaving at 15 pounds steam pressure for 20 minutes. Each of the flasks was inoculated with 1 ml. of a concentrated (four agar slant growths washed into 75 ml. of water) spore-mycelium suspension. Incubation was at 28°C. in the dark, unless otherwise noted. The period of incubation varied with the nature of the experiment. At the end of the desired incubation period, the growths were washed with distilled water on a filter cloth until free of extracellular metabolites, as indicated by the complete absence of glucose and ethyl alcohol in the washings. The washed mats were then pressed lightly to remove excess wash water and the moist tissue was introduced into a shaking bottle containing glass beads, and 15–25 ml. of M/20 KH_2PO_4 buffer at pH 4.85 for each mycelial mat used. The flasks were shaken vigorously until a uniform suspension was obtained as indicated by the ability to pipette easily with a 2.5 mm. bore pipette. Such suspensions gave unusually uniform results. Older growths (10 days) are more difficult to disperse unless they are minced finely before shaking. The dry weight of tissue per unit volume of suspension was determined for subsequent calculations.

Attempts were made to prepare cell-free preparations by grinding with sand in the cold with subsequent centrifugation; by freezing and grinding; and by pressing at very high pressures. In all cases, inactive preparations were obtained. The dried, washed tissue also shows no activity (CO_2 production) after re-suspension in a phosphate-glucose

solution. Boyland *et al.* (1937) showed that the reason for the inactivity of tumor extracts is to be found in the very rapid destruction of cozymase and adenylpyrophosphate which occurs on damage to the cells. Euler *et al.* (1936) found similar changes in acetone preparations of brain. A later communication will deal with the possible rôle of cozymase in *Fusaria* metabolism. In these studies, therefore, freshly prepared, washed suspensions of cells (as described above) were employed.

The respiratory and fermentative activities of the organisms were measured manometrically by means of Barcroft manometers in a water bath controlled to ± 0.02°C. Unless otherwise noted, experiments were carried out at 30°C. The manometers were shaken at 120 oscillations per minute. The cups and contents were adapted to the temperature of the bath for 10 minutes before the manometer taps were shut. Caustic was replaced frequently during long experiments where large volumes of CO_2 were evolved. By the direct, two manometer method (Dixon, 1934), the CO_2 evolved and the oxygen consumed could be estimated and the R.Q.; *i.e.*, CO_2 evolved/O_2 consumed could be determined. An atmosphere of air was used for the aerobic studies. For anaerobic experiments an atmosphere of nitrogen was used. From the dry weight of organism used the $Q^{air}_{O_2}$; *i.e.*, c.mm. of O_2 consumed per hour per milligram of dry weight was calculated. In the same way $Q^{air}_{CO_2}$ and $Q^{N_2}_{CO_2}$ were calculated.

EXPERIMENTAL AND DISCUSSION

Effect of Age of Growth on Endogenous (Respiratory) and Exogenous (Fermentative) Activity of Fusarium sp. H.

A number of flasks of the usual medium were inoculated with the concentrated spore-mycelium suspension and incubated at 28°C. in the dark. At desired intervals suitable numbers of flasks to give sufficient preparation were removed and a suspension was prepared for immediate use as described. When the preparation was not in use during an experiment, it was stored at 0°C. No preparations were stored for more than 8 hours. The experiment was carried out over a period of 7 days and estimations were made of hourly R.Q., $Q^{air}_{O_2}$, and $Q^{air}_{CO_2}$. In order to correlate the production of alcohol with the respiratory and fermentative activity of the organism, analyses of the alcohol content of the metabolism solutions on which the organism had been grown were carried out by the method of Friedemann and Klaas (1936). The results of the experiment are given in Table I.

From the results it is apparent (1) that in very young mats the $Q^{air}_{O_2}$ is extremely high, and with increase in the age of the growth the $Q^{air}_{O_2}$ falls markedly. (2) In the presence of added glucose, the $Q^{air}_{CO_2}$ and the R.Q. of young mats show an immediate marked increase which seems to indicate an immediate attack of the added glucose. With increase in age the response to added glucose is delayed, but definite after incubation with glucose for a short period. The incubation period is increased as the age of the mat increases. (3) In the course of the endogenous metabolism of very young

mats, the $Q_{O_2}^{air}$ and the $Q_{CO_2}^{air}$ fall markedly as the experiment progresses, so that after 4 hours, the $Q_{O_2}^{air}$ has fallen from 39.5 for the 1st hour to 13.1 for the 4th hour. The $Q_{CO_2}^{air}$ falls from 31.9 to 11.0. The exogenous metab-

TABLE I

Metabolic Activity of Resting Cell Suspensions of Fusarium sp. H. Prepared from Cultures of Varying Age

Time (hrs.)	Without added glucose			With added glucose		
	$Q_{O_2}^{air}$	$Q_{CO_2}^{air}$	R.Q.	$Q_{O_2}^{air}$	$Q_{CO_2}^{air}$	R.Q.
1 day cultures (alcohol content of growth medium 0.08 mg./ml.)						
1	39.5	30.7	0.78	34.4	63.7	1.85
2	30.3	25.7	0.84	38.2	56.3	1.49
3	19.3	15.7	0.81	38.9	55.1	1.42
4	13.1	11.0	0.84	36.1	56.0	1.55
2 day cultures (alcohol content of growth medium 0.55 mg./ml.)						
1	16.1	16.1	1.00	14.7	21.7	1.48
2	15.2	12.1	0.80	13.6	23.0	1.69
3	14.0	12.0	0.86	11.3	22.7	2.05
4	9.9	9.0	0.90	12.3	26.0	2.14
5	8.2	6.9	0.85	11.3	26.0	2.30
3 day cultures (alcohol content of growth medium 0.62 mg./ml.)						
1	13.9	14.0	1.01	14.1	19.2	1.36
2	15.5	14.1	0.91	15.0	22.6	1.50
3	14.3	12.7	0.89	14.3	26.7	1.85
4	12.9	11.9	0.92	13.2	26.1	1.97
5 day cultures (alcohol content of growth medium 0.80 mg./ml.)						
1	7.3	6.7	0.91	6.1	6.1	1.00
2	7.0	5.7	0.81	6.0	7.0	1.17
3	7.0	5.5	0.78	6.8	9.7	1.43
4	6.1	4.7	0.77	5.8	10.2	1.78
7 day cultures (alcohol content of growth medium 0.64 mg./ml.)						
1	6.5	6.0	0.93	5.9	6.2	1.05
2	6.2	5.3	0.86	5.7	6.5	1.14
3	6.2	5.3	0.87	5.7	7.4	1.30

olism (added glucose), however, shows no such decrease, remaining fairly constant throughout the duration of the experiment. This is analogous to the observations on young yeast cultures by Stier and Stannard (1936) where the progressive fall of endogenous activity is attributed to the progressive utilization of "endogenous substrate" and that the dissimilation of

carbohydrate stores is a respiratory process. (4) During the 1st day alcohol production is very low, which would indicate that endogenous metabolism is predominant, since analyses show that alcohol is not produced in endogenous metabolism but is produced during exogenous metabolism (Table II). This is also the case in yeast metabolism. After the first day there is a sudden marked increase in alcohol production which seems to be correlated with the stabilization of the endogenous metabolism, since the $Q_{O_2}^{air}$ (endogenous) during the course of an experiment now remains relatively constant. Again the analogy to the mechanism in living yeast is very pronounced.

TABLE II

Ethyl alcohol production in "nutrient" and "non-nutrient" media by *Fusarium* sp. *H.* resting cell suspensions which correspond to the exogenous and endogenous metabolism, respectively.

Suspension prepared from	Suspended in	Time of incubation and shaking	Alcohol production*
		hrs.	*mg./ml.*
3 day mats	Dextrose-phosphate	6	0.23
3 " "	Phosphate	6	0.00
4 " "	Dextrose-phosphate	5	0.17
4 " "	Phosphate	5	0.00

* Ethyl alcohol as analyzed by the method of Friedemann and Klaas (1936). Experiments were carried out at room temperature and alcohol determinations were made in triplicate on the suspension filtrates.

The Induction Period

It is apparent that for the study of growths older than 1 day an incubation period with glucose or any other substrate to be studied is necessary to make apparent any excess (exogenous) carbon dioxide production. Similar precautions must be taken with yeast where it is believed that either a reserve material must be built up (Willstätter and Rohdewald, 1937) or, the organism must be brought to a certain anabolic phase or level (Stier and Stannard, 1936). The occurrence of an induction period in the fermentation of glucose by living yeast has been observed by Willstätter and Rohdewald (1937) who found a marked increase in glycogen during this period and suggested that glucose is first transformed to glycogen before fermentation. Goda (1938) also observed the formation of glycogen in young yeast but observed that in old yeast there was a rapid fermentation of added glucose without a parallel formation of glycogen.

From the data in a typical experiment (Table III) it is evident that no appreciable *fermentable* reserve material is built up. It might be suggested that during the period of incubation the glucose is fragmented to smaller,

more available intermediates which are in solution and which then give rise to carbon dioxide. That this is not the case is evident from experiments where suspensions were incubated with glucose until the R.Q. was well above the endogenous R.Q., then thoroughly washed until free of any metabolites contained in the medium, and re-suspended in a fresh glucose-phosphate buffer solution. The R.Q. obtained after re-suspension was approximately equal to the R.Q. before washing free from the medium. When incubation is carried out in the presence of iodoacetate, CO_2 evolution and O_2 uptake are markedly inhibited due to long exposure to the poison.

TABLE III

Experiments Indicating the Direct Attack of Glucose by Fusarium sp. H.

	Experiment (1)	Experiment (2)
1. R.Q. without added substrate immediately after preparation of suspension	0.87	1.08
2. R.Q. with added glucose (0.07 M) immediately after preparation of suspension	0.92	1.06
3. R.Q. after shaking with glucose (0.07 M) for 3 hrs.	1.88	1.66
4. R.Q. after shaking with glucose (0.07 M) for 3 hrs. and then washing from glucose	1.02	—
5. R.Q. after shaking with glucose (0.07 M) for 3 hrs., washing away the metabolism solution, and replacing with glucose (0.07 M)	1.67	—
6. R.Q. after shaking with glucose (0.07 M) and 0.004 M iodoacetate for 3 hrs.	—	1.11
7. R.Q. after shaking with glucose (0.07 M) and 0.004 M iodoacetate for 3 hrs., then washing, and suspending in glucose-free buffer	—	1.00
8. R.Q. after treating as (7) except suspended in glucose after washing	—	1.03

The fermentative system is, however, completely inhibited. From the data in experiment 1 in Table III, it can be seen that there is a slight rise in R.Q. from 0.87 for the endogenous rate to a new endogenous value of R.Q. = 1.02 after shaking with glucose and then washing away the suspending medium. This might be accounted for by the presence of either a small amount of intracellular glucose or by other intracellular intermediate products of the breakdown of glucose. It is apparent therefore that no unlimited accumulation of intermediate products occurs but that there may be a constant and perhaps extremely low working level of such substances. That the excess carbon dioxide does not arise from a reserve material seems quite evident.

It has been found that under certain conditions of growth, *i.e.* when the flasks were sown with dilute spore-mycelium suspensions and incubated in

the light at 22–24°C. rather different growths were obtained which were characterized by the fact that the suspensions of old organisms (10–11 days) could attack glucose with formation of CO_2 directly (without an incubation period). Direct attack of added glucose by this type of growth as compared to the delayed attack by the usual growths must be due to differences in mat permeability rather than the necessity for building up cellular glycogen-like substances since in the absence of glucose the usual low endogenous R.Q. is obtained.

The Nature of the Endogenous and Exogenous Metabolism

The endogenous and exogenous mechanisms in *Fusaria* metabolism were investigated and shown to be similar to the mechanisms in yeast. These methods of study were employed: (1) carbon dioxide evolved aerobically and anaerobically with and without glucose, and (2) the effects of poisons such as sodium iodoacetate, potassium fluoride, and cyanide on the exogenous and endogenous mechanisms.

(*a*) *Aerobic and Anaerobic CO_2 Production*

From the results shown in Table IV, it is apparent that in the absence of substrate there is no significant anaerobic production of CO_2. However, with added glucose there is definite anaerobic CO_2 production which is equal to about 70 per cent of the added CO_2 produced aerobically in the presence of added glucose. This would indicate that the mechanisms are different, and also that a part of the increase in CO_2 production due to added glucose may involve an aerobic mechanism.

Experiments were carried out in the usual manner and varying concentrations of inhibitor added in M/20 phosphate to give the desired final concentration. In some instances the concentrations were increased as the experiments progressed. The results are indicated in Table V. It is apparent that both iodoacetate and fluoride in relatively low concentrations affect the exogenous metabolism without affecting the endogenous metabolism. Higher concentrations do affect the endogenous metabolism without disturbing the R.Q. Similar observations have been made on yeast cells by Stier and Stannard (1936). With certain concentrations of iodoacetate, the exogenous metabolism has been completely inhibited with no simultaneous effect on the endogenous metabolism. A series of experiments with fluoride and iodoacetate on the anaerobic CO_2 production shows a very marked decrease on the addition of these specific inhibitors (Table IV). Cyanide, on the other hand, markedly affects the respiratory mechanism but only slightly affected fermentation.

From these experiments it can be concluded that the exogenous and endogenous CO_2 producing-mechanisms are distinct, and in general, the situation in *Fusaria* is analogous to that in yeast.

TABLE IV

The Nature of the Anaerobic Carbon Dioxide-Producing Mechanism of Resting Cell Suspensions of Fusarium sp. H

1. Anaerobic production of CO_2 with and without dextrose

Experiment	Medium	CO_2 evolved c.mm./hr. (N_2 atmosphere)	$Q_{CO_2}^{N_2}$	$Q_{CO_2}^{air}$
1	Phosphate	15	Negligible	34.2
	Dextrose-phosphate	257	27.8	63.7
2	Phosphate	0	0.0	30.7
	Dextrose-phosphate	224	22.0	54.1

2. Effect of iodoacetate on the anaerobic (nitrogen atmosphere) production of CO_2

Molarity of IAA	c. mm. CO_2 evolved/hr.	Per cent inhibition
Control	224	—
0.0040	67	70
0.0065	26	88

3. Effect of fluoride on the anaerobic (nitrogen atmosphere) production of CO_2

Molarity of KF	c. mm. CO_2 evolved/hr.	Per cent inhibition
Control	265	—
0.0040	133	50
0.0400	31	87

Fermentation of Various Carbon Sources by Fusarium sp. H.

A study has been made to determine the ability of the organism to attack a variety of common carbon sources with the production of exogenous CO_2, (1) when grown on glucose and suspended in a medium containing a carbon source to be studied or, (2) when grown on a carbon source other than glucose and suspended in a medium containing that carbon compound or another. The organisms were grown in the usual manner on 1 per cent solutions of glucose or another carbon source, and suspensions were prepared as described previously. The results of the experiments are summarized in Table VI. It is evident that (1) the glucose-dissimilating mechanism is residual in all growths, no matter what the source of carbon for growth has been, and may be called constitutive. When grown on non-hexose sources of carbon, the residual glucose-dissimilating power is very small; but when grown on any of the hexoses investigated, the glucose-

TABLE V

*Effect of Cyanide on the Respiratory Activity**

Inhibitor	O_2 uptake c. mm./hr.	O_2 uptake Inhibition	CO_2 evolution c. mm./hr.	CO_2 evolution Inhibition	R.Q.
		per cent		*per cent*	
Control	247	—	270	—	1.09
0.00025 M KCN	217	16.4	251	7	1.16
0.0005	191	22.7	242	10.4	1.27
0.0020	156	36.9	216	19.6	1.38
0.0040	137	44.5	167	38.1	1.22
0.0080	123	50.0	153	43.3	1.24
0.0120	60	75.8	77	71.4	1.28
Effect of cyanide on the fermentative activity†					
Control	165	—	412	—	2.49
0.00025 M KCN	131	20.6	440	Accel.	3.38
0.0005	88	46.7	394	4.4	4.47
0.0010	90	46.5	369	10.4	4.10
0.0020	94	43.1	301	26.9	3.20
0.0040	73	55.7	235	43.0	3.22
Effect of potassium fluoride on the respiratory activity					
Control	148	—	136	—	0.93
0.0012 M KF	148	0	140	0	0.95
0.0018	148	0	138	0	0.94
0.0024	148	0	133	0	0.90
0.0048	158	0	140	0	0.89
0.0056	148	0	132	0	0.89
0.0090	127	14.2	117	14.0	0.92
0.0180	111	25.0	116	14.0	1.04
Effect of fluoride on the fermentative activity					
Control	284	—	625	—	2.20
0.0012 M KF	290	0	502	19.7	1.73
0.0024	288	0	400	36.0	1.39
0.0036	255	10.2	372	40.5	1.46
0.0048	245	13.7	333	46.7	1.36
Effect of sodium iodoacetate on the respiratory activity					
Control	195	—	190	—	0.97
0.0010 M IAA	190	0	192	0	1.01
0.0020	187	0	187	0	1.00
0.0030	197	0	190	0	0.96
0.0040	189	0	188	0	1.00
0.0060	167	14.3	160	15.8	0.96
0.0070	144	26.2	137	27.9	0.95
Effect of iodoacetate on the fermentative activity					
Control	180	—	324	—	1.80
0.0010 M IAA	182	0	260	19.8	1.43
0.0020	187	0	214	34.0	1.15
0.0040	179	0	171	53.0	0.95

* No added glucose.

† 1 per cent glucose.

dissimilating power is equal to that of the growths when grown on glucose itself. This is analogous to the observations on bacteria by Hegarty (1939) and Karström (1938). (2) Growths on glucose will attack glucose, mannose, fructose, but not galactose. Fructose is always attacked least vig-

TABLE VI

Composite Table of R.Q. of Fusarium sp. H. on Various Substrates

Tested on	Grown on							
	Glucose*	Glucose	Galactose*	Galactose + glucose	*l*-Xylose	*l*-Arabinose	Glycerol	Lactate
No substrate	0.95	0.88	1.58	1.20	1.00	0.87	0.92	0.91
Glucose	1.75	1.84	2.52	2.30	1.20	1.17	1.24	1.30
α−Glucose	1.60	—	—	2.25	—	—	—	—
Mannose	1.71	1.67	2.39	2.20	—	—	—	—
Galactose	0.95	0.90	2.33	2.00	0.97	0.88	0.90	0.88
Fructose	1.33	1.37	1.67	1.41	1.02	0.96	0.93	0.95
Xylose	0.95	0.87	—	1.16	0.98	0.88	0.89	0.92
Arabinose	0.93	0.89	—	—	0.89	0.89	0.88	0.96
Lactose	0.83	0.89	1.64	1.26	—	—	—	—
Sucrose	1.14	1.09	1.60	—	—	—	—	—
Maltose	0.93	0.89	—	1.19	—	—	—	—
Mannitol	0.89	0.90	—	—	—	—	—	—
Glucose+mannose	1.70	—	—	2.24	—	—	—	—
Glucose+galactose	1.75	1.80	2.55	2.30	—	—	—	—
Glycerol	0.61	0.59	—	—	0.50	0.21	0.64	0.62
α−glycerol PO_4	0.51	0.61	—	0.53	—	—	—	—
Hexose diphosphate	—	0.95	—	—	0.93	0.90	—	—
Pyruvate	1.35	1.44	1.88	—	1.38	1.43	1.31	1.47
Pyruvate+glucose	2.08	2.17	2.80	—	—	—	—	—
Succinate	0.96	0.89	1.61	—	1.00	0.92	0.87	0.88
Lactate	0.84	0.78	—	—	0.68	0.72	0.77	0.68
Acetate	0.96	0.86	—	—	—	—	0.89	0.89
Ethyl alcohol	0.87	0.84	—	—	—	—	—	—
Salacin	0.94	0.87	—	—	—	—	—	—
Citrate	0.96	0.92	—	—	—	—	—	—
Phosphoglyceric acid	—	0.89	—	—	—	—	—	—

* Cultures attacked glucose directly.

orously. When grown on galactose, the organism can attack the usual carbohydrates and galactose. Glucose, however, always seems to be more vigorously attacked than galactose. Similar studies on galactose adaptation have been made with yeast by Stephenson and Yudkin (1936) and on bacteria by Stephenson (1939). It is also interesting to note that when the organism is grown on galactose or a mixture of galactose-glucose the endogenous R.Q. is considerably higher. This is consistent with the concept that certain sugars are "growth" sugars rather than "fermentable" sugars.

Of the common intermediary metabolites investigated only pyruvate is attacked to give added CO_2 evolution. This is due to the presence of a carboxylase system, which will not be discussed in this communication, and suggests that pyruvic acid is perhaps an intermediate in the dissimilation of glucose by *Fusaria*. It would be expected that pyruvic acid would be attacked at least as vigorously as glucose, but that this does not appear to be the case, from the R.Q. values for 1 hour, may be due to the toxic effect of accumulated acetaldehyde since in the first few minutes the rate of CO_2 evolution from pyruvic acid is extremely high and falls markedly as the reaction progresses. It is also apparent that when the organisms are grown on non-hexose sources of carbon they could not attack any substrate but the hexoses or pyruvic acid with direct evolution of CO_2 under these experimental conditions.

The Nature of the Hexose-Dissimilating Mechanisms

The identity of the mechanisms involved in the direct attack of glucose, mannose, and galactose has been confirmed by addition experiments where the dissimilation of the substrates was investigated separately and then when present together. No additive effects were observed as is indicated from the data in Table VI. It would appear, therefore, that a single dissimilating mechanism is involved for these substrates and that the mechanism is adaptive for galactose dissimilation.

SUMMARY

1. *Fusarium tricothecoides* was selected for a study of the respiratory and fermentative activities of *Fusaria*. "Resting cell" suspensions were investigated by the Barcroft manometric technique.

2. The results of the investigation indicate clearly that the mechanism of endogenous metabolism (respiration) is distinct from the exogenous mechanism (fermentation). Anaerobically no significant CO_2 production is apparent without added substrate. In the presence of glucose the anaerobic CO_2 evolution is practically equal to the added CO_2 evolved aerobically in the presence of added glucose.

Low concentrations of iodoacetate or fluoride selectively poison the exogenous mechanism but do not affect the endogenous mechanism.

Alcohol is not produced in the course of endogenous metabolism, but is produced in the presence of added glucose.

3. A study of the metabolism of the organism throughout its entire growth phase from 1 to 7 days has been made.

4. The ability of suspensions of *Fusarium* sp. *H.*, obtained by growth on a variety of common substrates, to attack a large number of carbon

sources with the production of exogenous CO_2 was determined. It is found that organisms grown on glucose will attack only glucose, mannose, and fructose, but none of the common intermediary metabolites except pyruvic acid. Organisms grown on galactose attack galactose, as well as the other hexoses, indicating an adaptive mechanism.

5. An identical mechanism for the dissimilation of glucose, mannose, and galactose is indicated since no additive effects with these substrates were observed. Growths on non-hexose carbon sources attack glucose slightly under the experimental conditions with the evolution of CO_2, but do not attack any other substrate. This would indicate a residual glucose-dissimilating mechanism in all growths investigated.

6. Striking similarities between the general metabolism of resting suspensions of *Fusarium* sp. *H.* and resting suspensions of yeast cells are apparent.

LITERATURE CITED

Birkinshaw, J. H., Charles, J. H. V., Raistrick, H., and Stoyle, J. A. R., Studies in the biochemistry of microorganisms. Part V, *Phil. Tr. Roy. Soc. London, Series B*, 1931, **220,** 93.

Boyland, E., Boyland, M. E., and Greville, G. D., The effect of cozymase on glycolysis in tumour extracts, *Biochem. J.*, London, 1937, **31,** 461.

Dixon, M., Manometric methods, Cambridge University Press, 1934.

Euler, H. von, Günther, G., and Vestin, R., Glycolyse und Phosphatumsatz in zellfreien Gehirnextrakten normaler Säugetiere, *Z. physiol. Chem.*, 1936, **240,** 265.

Friedemann, T., and Klaas, R., The determination of ethyl alcohol, *J. Biol. Chem.*, 1936, **115,** 47.

Goda, T., Kann lebende Hefe Hexosen direkt vergären? *Biochem. Z.*, Berlin, 1938, **298,** 431.

Hegarty, C. P., Physiological youth in enzyme formation, *J. Bact.*, 1939, **37,** 145.

Karström, H., Enzymatische Adaptation bei Microorganismen, *Ergebn. Enzymforsch.*, 1938, **7,** 350.

MacFarlane, M. G., The phosphorylation of carbohydrates in living cells, *Biochem. J.*, London, 1939, **33,** 565.

Nord, F. F., Dammann, E., and Hofstetter, H., Ist bei der alkoholischen Zuckerspaltung in der Zelle die Phosphorylierung zwangsläufig? Beitrag zur Biochemie von *Fusarium lini B.*, *Biochem. Z.*, Berlin, 1936, **285,** 241.

Nord, F. F., Enzymatische Umsetzungen durch *Fusarien*—Beitrag zum mechanismus der Alkoholischen Gärung, *Ergebn. Enzymforsch.*, 1939, **8,** 149.

Pritham, G. H., and Anderson, A. K., Carbohydrate metabolism of *Fusarium lycopersici* on glucose, *J. Agric. Research*, 1937, **55,** 937.

Sherbakoff, C. D., *Fusaria* of potatoes, Ithaca, Cornell University Press, 1915.

Stephenson, M., Bacterial metabolism, London, Longmans Green and Co., 1939.

Stephenson, M., and Yudkin, J., Galactozymase considered as an adaptive enzyme, *Biochem. J.*, London, 1936, **30,** 506.

Stier, T. J. B., and Stannard, J. N., A kinetic analysis of the endogenous respiration of bakers' yeast, *J. Gen. Physiol.*, 1936, **19**, 461.
Stier, T. J. B., and Stannard, J. N., The metabolic systems involved in dissimilation of carbohydrate reserves in bakers' yeast, *J. Gen. Physiol.*, 1936, **19**, 479.
White, M. G., and Willaman, J. J., *Fusarium lini* and the pyruvic acid theory of alcoholic fermentation, *Biochem. J.*, London, 1928, **22**, 592.
Willstätter, R., and Rohdewald, M., Über die erste Phase der Gärung durch Hefe, *Z. physiol. Chem.*, 1937, **247**, 269.
Wollenweber, H. W., Studies on the *Fusarium* problem, *Phytopathology*, 1913, **3**, 24.

ANAEROBIC GLYCOGENOLYSIS IN THE MUSCLES OF RANA PIPIENS LIVING AT LOW TEMPERATURES

By A. N. BARGER and R. E. JOHNSON

(*From the Fatigue Laboratory, Harvard University, Boston*)

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When attempts failed (16) to produce glycolytically active extracts from the muscles of winter frogs stored at 4°C., and when lactate production at 0°C. by their gastrocnemii could not be detected (12, 17) we began to study the glycogen metabolism during fatigue of the muscles from such frogs. Previous workers have shown alterations in the carbohydrate metabolism of frog muscles with low glycogen contents, and also seasonal variations in the carbohydrate metabolism of frogs.

If sartorii and gastrocnemii of *R. temporaria* and *R. esculenta* have low carbohydrate reserves, they can contract aerobically on sources of energy only partly carbohydrate (30, 10). When mammalian muscles are rendered low in glycogen by the use of insulin or thyroid, rigor mortis in them is not accompanied by glycogenolysis or by lactate production (15). Olmsted and his colleagues (31, 32) were the first to use insulin convulsions for rendering muscles of *R. pipiens* and *R. catesbiana* nearly free of glycogen; muscles so prepared contracted anaerobically without glycogenolysis or lactate production. Ochoa (30) and Gemmill (10) extended these observations to *R. temporaria* and *R. esculenta*.

It has been known for a long time that there is marked seasonal variation in the carbohydrate metabolism of frogs' tissues. Schiff (36) in 1859 observed that there is little postmortem glycogenolysis at 23°C. by the livers of frogs caught in the winter. Grode and Lesser (13) set up uninjured livers and muscles of frogs for 4 hours at 22-23°C. in Ringer's solution under aerobic conditions. In November and December such preparations showed only very slight decreases in glycogen, despite initial concentrations of 0.75 to 1.24 per cent. At other times of the year, decreases of 15–20 per cent of the initial value were observed. If in November or December the organs were cut or severely injured, marked glycogenolysis took place. Lesser (21) analyzed whole frogs for glycogen. In the summer, anaerobiosis of 2 hours led to marked glycogenolysis. A subsequent 24 hours of aerobiosis led to restitution of most of the glycogen. In November and December, anaerobiosis of 2 hours did not produce glycogenolysis.

This could be produced by repeated periods of anaerobiosis but aerobiosis did not result in restitution.

Lesser (22) described semiannual periods in a frog's life: the glycogen-stable period of winter, and the glycogen-labile of summer and fall.

Laquer (19, 20) studied lactate production by chopped frog muscles incubated in buffered media, of which phosphate buffers were found to be the most favorable to glycolysis. At 45°C., the muscles of summer frogs produced large amounts of lactate from added hexose diphosphate, starch, or glycogen, but the muscles of winter frogs did not produce lactate from added substrates. When winter frogs were kept alive for several days at temperatures between 22 and 27°C., their muscles gained the ability to produce lactate from added glycogen.

The present experiments were designed to show whether prolonged life at 4°C. altered the glycolytic capacity of frog gastrocnemii, either by depleting carbohydrate reserves, or, more fundamentally, as in the experiments of Lesser (22) and Laquer (19, 20), by altering their glycolytic capacity.

Methods

The experiments were done on two separate batches of frogs in the summers of 1939 and 1940. *R. pipiens* from northern Vermont, caught in March or April, were kept without food at 4°C. in a few milliliters of tap water that was changed every 2 days. They were stored in this way from 7 to 10 weeks before use. Any frog showing clinical signs of disease, especially "red leg," was discarded.

For each experiment, a frog was allowed to rest alone for several hours at 4°C. One operator then gently held it over a paper cutter and extended its hind legs. The second operator chopped off both its legs with a single stroke at the pelvic girdle, the first laid the legs on ice covered with moist gauze and killed the frog. The second dissected out both gastrocnemii with ice cold instruments, and both set up the muscles in the apparatus for anaerobiosis and stimulation. The usual elapsed time from the stroke that cut off the legs to installation in an atmosphere of nitrogen was 3 minutes.

The apparatus in which the muscle was stimulated consisted of a vertical glass tube 15 cm. long and 4 cm. in diameter, tightly fitted with one-holed stoppers. The muscle was supported in the chamber by a small brass clip at each end, one being firmly applied to the lower stopper, the upper to a fine thread leading to an isotonic myograph,[1] counterbalanced and backstopped. Moist nitrogen, containing less than 0.04 per cent oxygen, was fed from a saturating chamber through a glass tube in the lower stopper, and out through the upper. A thermometer was fastened to the upper stopper in each chamber. The muscles were stimulated directly through the brass clips by make shocks from an inductorium. The interval between stimulations was from 18 to 25 seconds in different experiments. This long interval was used to avoid any possibility of the muscle warming during the experiment. The load was adjusted to give a convenient excursion on the kymograph. The primary voltage was three, and the twitches were kept maximal

[1] Obtainable from the Harvard Instrument Co.

by adjustment of the secondary coil every 10 minutes. The muscles were stimulated until they would not respond even to the strongest stimulus. This was usually within 2 hours. Control muscles were treated like stimulated in every way but stimulation.

Some of the early experiments were carried out at room temperatures from 25 to 29°C., but the control values were so high, because of heat glycolysis, that all the later experiments were done at 0°C., by keeping both the stimulation chamber and the nitrogen moistener immersed in a wide-mouthed insulated jug filled with cracked ice. The temperature difference between the two chambers that contained the control muscle and the stimulated was never more than ½°C. The muscles did not change in water content in periods up to 2 hours.

When the stimulated muscle was completely fatigued, finely powdered carbon dioxide was poured into the chamber and into the control chamber at such a time that both muscles would have the same period of anaerobiosis. The frozen muscles were then stored in air-tight stoppered tubes surrounded by solid carbon dioxide. The water, glycogen, and lactate content of muscles stored in this way did not change in a week. Samples for estimation of water, glycogen, and lactate were taken by cutting the frozen muscle in a direction parallel with the fibers, into two large pieces and one small piece. The gastrocnemii averaged about 500 mg. in weight. For water determinations, about 50 mg. was used; for glycogen and lactate, about 200 mg.

Water determinations were made by drying the muscle for 24 hours at 110°C.

Glycogen was estimated by the method of Good, Kramer, and Somogyi (11) with slight modification. The tissue was hydrolyzed by 1 ml. of 30 per cent KOH in 15 cc. graduated conical centrifuge tubes fitted at the top with spherical air condensers seated in constrictions, and all subsequent manipulation of the glycogen was done in these tubes. When the glycogen was precipitated in 60 per cent alcohol, the mixture was vigorously stirred by a fine platinum wire fitted to an electric stirrer. This maneuver seemed to improve the precipitation of the glycogen. After the glycogen was hydrolyzed to glucose in 2.2 per cent HCl at 100°C. for 2½ hours, the pH was adjusted to 10 with thymol blue and 5 per cent KOH. The titratable acidity to pH 10.3 of the aliquots was not more than 0.01 cc. of N NaOH, as Forbes and Andreen-Svedberg (8) recommend. Glucose was estimated by the method of Folin and Wu (7). Brominated molybdate was not used, because the reagent blank with unbrominated, even after 2 weeks, was negligible. Each run of glycogen estimations contained suitable reagent blanks and a standard solution of pure glycogen. The blanks were always almost colorless, and recovery of added amounts of glycogen corresponding to the amount in the muscle samples, ranged from 99 per cent to 103 per cent of the amount added.

Muscle extracts for lactate estimations were prepared with trichloroacetic acid by the technique of Newman (29), and aliquots were analyzed by the Edwards (4) modification of the method of Friedemann, Cotonio, and Shaffer (9). Each run of lactate estimations contained suitable reagent blanks and solutions of pure lithium lactate. Recoveries of added lactate in amounts comparable to those present in the samples of muscle ranged from 97 per cent to 103 per cent, and the blanks were of the same order as Wendel's (37).

Control Experiments

Blanks were run on the surgical gauze, solid carbon dioxide, and nitrogen. They were negative for lactate and glycogen. The solid carbon dioxide

used in freezing several fatigued muscles was collected. It was negative for lactate and glycogen. The materials used therefore neither added blanks nor abstracted glycogen and lactate from the muscles.

The water content of 100 of the muscles used in the experiments was estimated. The mean was 80 per cent, S.D. 2.1 per cent. The data are not included, and no correction was made from one experiment to the next, since the water contents were practically the same in all muscles.

The resting value of lactate was estimated for twenty muscles from legs frozen as soon as they were chopped off. The mean value, in milligrams of lactic acid per 100 gm. muscle, was 17, S. D. 13. The average of the control muscles used in 53 experiments run at 0°C. was 25 mg. lactic acid per 100 gm. muscle, S. D. 17. These muscles had all been dissected out, clipped in the apparatus, and exposed to nitrogen for periods up to 2 hours. The control values in 1940 were lower than in 1939, probably because of increased skill in handling the frogs. We concluded that our handling of the frogs and muscles was satisfactory, and that nothing in the methods gave falsely high values for lactate.

The assumption has to be made in experiments such as these that the level of a given substance in the control muscle at any given time may be taken as that which the fatigued muscle would have had at that time if it had not been stimulated. This assumption was shown to be valid for glycogen and lactate by taking both gastrocnemii from each of twenty-five frogs, and treating the pairs exactly alike, in lots of four pairs. Those of the first lot were frozen as soon as they had been dissected out. Those of the second lot were set up in the apparatus and frozen at once. Those of the third lot were set up for 2 hours anaerobically at 0°C. before being frozen. Those of the last lot were set up anaerobically for 2 hours at 25°C. before being frozen. The differences between paired muscles averaged 17 mg. lactic acid per 100 gm. muscle and 62 mg. glycogen per 100 gm. muscle. The differences were independent both of the initial glycogen content and of the previous treatment of the muscle before it was frozen. In three cases the difference in glycogen fell just outside the limits established by Kerly (18) for frogs and by Cori (3) for rats. In Table I, significant changes in glycogen and lactate are considered to be 62 and 17, respectively.

Experiments with Fatigued Muscles

The results of the experiments in which muscles were stimulated anaerobically to complete fatigue (Table I) were variable. About one-half the muscles showed decreases in glycogen and increases in lactate in the range one usually finds with autumn frogs, that is, changes of 100 mg. or more

TABLE 1

Changes in Glycogen and in Lactate during Anaerobic Contraction at 0°C. to Complete Exhaustion

Glycogen is expressed as milligrams of glucose per 100 gm. muscle, wet weight. Lactate is expressed as milligrams of lactic acid per 100 gm. muscle, wet weight.

Experiment No.	Days kept, No.	Contractions, No.	Glycogen		Lactate		Loss in glycogen (control minus twitch)	Gain in lactate (twitch minus control)
			Control	Twitch	Control	Twitch		
A. Eleven experiments of 1939 at 0°C. without much glycogenolysis								
			mg. per cent	*mg. per cent*	*mg. per cent*	*mg. per cent*	*mg. per cent*	*mg. per cent*
20	50	238	494	443	89	120	51	31
28	52	233	142	63	44	55	79	11
29	52	219	346	361	34	117	−15	83
32	52	139	388	378	48	63	10	15
33	53	280	520	520	8	104	0	96
50	22	214	670	685	9	86	−15	77
51	22	160	623	600	68	61	23	−7
60	50	204	370	354	84	82	16	−2
61	50	227	552	540	16	90	12	74
70	57	222	130	105	7	48	25	41
72	57	202	359	320	12	54	39	42
Average.......		213	417	397	38	80	20	42
B. Sixteen experiments of 1940 at 0°C. without much glycogenolysis								
88	25	144	755	790	31	29	−35	−2
89*a*	74	140	475	465	14	22	10	8
89*b*	74	140	430	390	20	66	40	46
90	75	288	670	620	20	20	50	0
91	75	153	600	670	40	26	−70	−14
92*b*	75	144	1160	1090	19	81	70	62
93	76	232	350	310	13	82	40	69
94	76	110	560	620	16	58	−60	42
95	76	140	670	580	14	33	90	19
99*a*	76	130	750	800	14	38	−50	24
99*b*	76	109	18	20	14	8	−2	−6
101	77	400	465	425	25	140	40	115
102	77	343	220	195	18	109	25	91
103	77	390	605	640	18	105	−35	87
104	78	360	670	620	13	122	50	109
105	78	207	235	240	16	55	−5	39
Average......		214	540	530	19	62	10	43
C. Twenty-six experiments of 1939 and 1940 at 0°C. with significant glycogenolysis								
Muscle pairs with highest difference		386	720	310	25	176	410	151
Muscle pairs with lowest difference		140	145	50	26	66	95	40
Average for all muscles showing significant glycogenolysis........		249	618	475	22	116	143	94

per 100 gm. of muscle. About one-half of them showed no significant decrease in glycogen, and about one-seventh of them showed no significant change in either glycogen or lactate; *viz.*, experiments 32, 51, 60, 88, 89*a*, 90, 91, and 99*b*. Only the highest, lowest, and average values are given for the experiments that showed significant changes in both glycogen and lactate. The data for the other experiments are given in detail. One explanation for the extreme variability of the results is perhaps suggested by Laquer's work (19) showing that warming winter frogs for a few days restores the ability of their muscle tissue to break down added glycogen. The frogs used in the present experiments were caught by a dealer in Vermont, shipped to Boston, and stored in an animal farm until we bought them. There are no means of telling how much handling and warming they received between their native habitat and our refrigerator.

In agreement with the work of others (18 and 23–27) there was no stoichiometric relation between glycogenolysis and lactate production. Three times as many muscles showed no change in glycogen as showed no significant increase in lactate. This suggests that many of the muscles used preformed carbohydrate intermediaries to produce lactate, but did not break down glycogen, even though it was present.

For each of the experiments of 1939, a calculation was made of the quantity:

$$\frac{\text{(Sum of the heights of all contractions)} \times \text{(weight lifted)}}{\text{(Weight of muscle in grams)}}$$

It gives a rough index of the capacity of a muscle to do physical work. These data are not included, because there was no simple relation between glycolysis and work done. Some of the muscles that did the most work showed no change in either glycogen or lactate, and some of those that did the least showed the most glycolysis. Further, there was no correlation between the capacity of the muscle to do work and its glycogen content. Some of the muscles that did very little work had glycogen contents of over 500 mg. per 100 gm. of muscle, and some of those that did the most work had the least glycogen.

The effect of temperature on these experiments is not clear. Of fifteen experiments at 25 to 29°C., eight showed no extra glycogenolysis during fatigue, over and above the heat glycogenolysis that was proceeding. The control lactate values were so high because of heat glycolysis that all of the later experiments were made at 0°C. Some of the experiments cited by Fletcher and Hopkins (6), Meyerhof (26–28), Hartree and Hill (14), and Peters (34), taken together, imply that the lactate production of fatigue

is less at 0° than at 25°C., but in our experiments at 25 to 29°C. two muscles failed to produce more lactate in fatigue than the lactate produced by heat glycolysis.

The one result reported here that differs considerably from previous work has to do with the relation between glycogen level and capacity to glycolyze. Olmsted and his colleagues (31, 32) found two moribund *R. catesbiana* whose muscles had practically no glycogen and contracted without glycolysis. None of the normal frogs of Ochoa (30) and of Gemmill (10) had muscles that contracted anaerobically without glycolysis, although some of those treated with insulin did so. These three workers and Hoet and Marks (15) consider that a necessary and sufficient condition for anaerobic contraction without glycolysis is depletion of the muscle stores of carbohydrate. Gemmill (10) implied that the capacity of the muscle to do work anaerobically is directly proportional to the carbohydrate level, the important carbohydrate being glycogen. In the present experiments, there was no evident correlation between the glycogen content of the muscle and its failure to glycolyze. Some of the muscles that did not glycolyze had over 500 mg. glycogen per 100 gm. muscle, namely, experiments 51, 88, 90, and 91. Further, even in those muscles that did glycolyze, there was no correlation between the level of glycogen and the amount of change either in glycogen or in lactate. It should be emphasized that some of the muscles twitched 150 times or more, and could do a considerable amount of work with either a high or a low glycogen content. Evidently they had a store of glycogen, but did not use it.

The source of energy for those muscles that did not glycolyze is puzzling. Muscles poisoned with iodoacetate still break down glycogen (25) and split abnormally large amounts of phosphocreatine (25). Ochoa (30) and Gemmill (10) found a few muscles from frogs treated with insulin that did not glycolyze anaerobically and did not split abnormally large amounts of phosphocreatine. Palazzolo (33) first showed that fatty acids disappear when the muscles of frogs and of hibernating hedgehogs become fatigued. There is a similarity between our frogs and Chambers' (2) dogs which, in the third stage of starvation, cannot utilize carbohydrate, even if it is available. Buchwald and Cori (1) have demonstrated a disappearance of fatty acids from frog muscles stimulated to exhaustion. The Meyerhof school (30, 10) say that aerobic contraction by isolated muscles of frogs can take place on sources of energy not all carbohydrate, and suggest fat as a direct source of energy for contraction. Our results indicate that anaerobic contraction under special circumstances can take place without breaking down glycogen.

Addendum.—Unpublished work of Edwards and Dill (5) bears on the present experiments. They went surf fishing on October 11, 1931, in Buzzard's Bay. The water temperature was near 10°C. and there was snow on the ground. They caught a small shark and three skates, pulled them struggling through the surf, killed them, and froze sections of the dorsal muscles in solid carbon dioxide. The blood lactate of the shark was 84 mg. lactic acid per 100 cc., of the skates, 42, 15, and 18. The muscle lactates of these skates were 42, 25, and 31 mg. of lactic acid per 100 gm. muscle. That is, under identical conditions, the blood lactate of the shark was high, but the blood and muscle lactates of two of the skates were at resting levels. These few observations suggest that the muscles of these skates were contracting under conditions that were essentially anaerobic, and yet were not producing much lactate.

SUMMARY

1. A considerable proportion of *R. pipiens* caught in the spring and stored without food for several weeks at about 4°C. had gastrocnemii that did not break down glycogen when they contracted anaerobically to complete exhaustion. A smaller number of the same muscles did not produce lactate.

2. There was no evident relation between failure to break down glycogen and the glycogen content of such muscles, some of which had more than 500 mg. of glycogen per 100 gm. of tissue.

3. The hypothesis of Meyerhof and his followers that aerobic contraction of frog muscles may at times take place with sources of energy other than carbohydrate is therefore extended to include anaerobic contraction.

BIBLIOGRAPHY

1. Buchwald, K. W., and Cori, C. F., *Proc. Soc. Exp. Biol. and Med.*, 1931, **28**, 437.
2. Chambers, W. H., *Physiol. Rev.*, 1938, **18**, 248.
3. Cori, G., *J. Biol. Chem.*, 1932, **96**, 259.
4. Edwards, H. T., *J. Biol. Chem.*, 1938, **125**, 571.
5. Edwards, H. T., and Dill, D. B., personal communication.
6. Fletcher, W. M., and Hopkins, F. G., *J. Physiol.*, 1907, **35**, 247.
7. Folin, O., *J. Biol. Chem.*, 1929, **82**, 83.
8. Forbes, W. H., and Andreen-Svedberg, A., *Skand. Arch. Physiol.*, 1934, **70**, 168.
9. Friedemann, T. E., Cotonio, M., and Shaffer, P. A., *J. Biol. Chem.*, 1927, **73**, 335.
10. Gemmill, C. L., *Biochem. Z.*, Berlin, 1932, **246**, 319.
11. Good, C. A., Kramer, H., and Somogyi, M., *J. Biol. Chem.*, 1933, **100**, 485.
12. Goodman, R., Harvard Senior Thesis, unpublished, 1939.
13. Grode, J., and Lesser, E. J., *Z. Biol.*, 1913, **60**, 371.
14. Hartree, W., and Hill, A. V., *J. Physiol.*, 1921, **55**, 133.
15. Hoet, J. P., and Marks, H. P., *Proc. Roy. Soc. London, Series B*, 1926, **100**, 72.
16. Jacobstein, S. E., Harvard Senior Thesis, unpublished, 1938.
17. Johnson, R. E., and Barger, A. N., unpublished observations.
18. Kerly, M., *Biochem. J.*, London, 1931, **25**, 671.

19. Laquer, F., *Z. physiol. Chem.*, 1921, **116**, 169.
20. Laquer, F., *Z. physiol. Chem.*, 1922, **122**, 26.
21. Lesser, E. J., *Z. Biol.*, 1913, **60**, 388.
22. Lesser, E. J., *Münch. med. Woch.*, 1913, **60**, 341.
23. Lundsgaard, E., *Biochem. Z.*, Berlin, 1930, **217**, 162.
24. Lundsgaard, E., *Biochem. Z.*, Berlin, 1934, **269**, 308.
25. Lundsgaard, E., *Ergebn. Enzymforsch.*, 1933, **2**, 179.
26. Meyerhof, O., *Arch. ges. Physiol.*, 1920, **182**, 232.
27. Meyerhof, O., *Arch. ges. Physiol.*, 1920, **182**, 284.
28. Meyerhof, O., *Arch. ges. Physiol.*, 1920, **185**, 11.
29. Newman, E. V., *Am. J. Physiol.*, 1938, **122**, 359.
30. Ochoa, S., *Biochem. Z.*, Berlin, 1930, **227**, 116.
31. Olmsted, J. M. D., and Harvey, J. M., *Am. J. Physiol.*, 1927, **80**, 643.
32. Olmsted, J. M. D., and Coulthard, H. S., *Am. J. Physiol.*, 1928, **84**, 610.
33. Palazzolo, G., *Arch. fisiol.*, 1913, **11**, 558.
34. Peters, R. A., *J. Physiol.*, 1913, **47**, 243.
35. Potter, V. R., and Elvehjem, C. A., *J. Biol. Chem.*, 1936, **114**, 495.
36. Schiff, R., quoted by Lesser, E. J., Untersuchungen über die Zuckerbestimmung in der Leber, Würzburg, 1859, 12.
37. Wendel, W. B., *J. Biol. Chem.*, 1933, **102**, 47.

SOME EFFECTS OF IODINE AND OTHER REAGENTS ON THE STRUCTURE AND ACTIVITY OF TOBACCO MOSAIC VIRUS

By M. L. ANSON AND W. M. STANLEY

(From the Laboratories and the Department of Animal and Plant Pathology of The Rockefeller Institute for Medical Research, Princeton, New Jersey)

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The SH groups of denatured egg albumin give a pink color with nitroprusside (Heffter, 1907; Arnold, 1911) and reduce porphyrindin (Kuhn and Desnuelle, 1938). Native egg albumin does not give these characteristic SH reactions. Despite the fact that native egg albumin does not reduce porphyrindin, the SH groups of egg albumin or their precursors can be abolished by reaction of the native form of egg albumin with iodine (Anson, 1940; 1941).

Tobacco mosaic virus is an SH protein of the egg albumin type, since denatured but not native tobacco mosaic virus gives a pink color with nitroprusside and reduces porphyrindin (Stanley and Lauffer, 1939) and since the SH groups of tobacco mosaic virus, as shown by the present experiments, can be abolished by reaction of the native form of the virus with iodine. Iodine is the only reagent known to abolish the SH groups of egg albumin and tobacco mosaic virus by reaction with the native form of these proteins. The observations that denatured tobacco mosaic virus has SH groups and that these groups or their precursors can be abolished by reaction of the native form of the virus with iodine suggested the study of the effect of iodine on the activity of tobacco mosaic virus. It was found in the present work that the SH groups of tobacco mosaic virus can be abolished by iodine without any change in the activity of the virus, as shown by the number of lesions produced by a given amount of modified virus on *Nicotiana glutinosa* plants or by the symptoms produced in Turkish tobacco plants.

Tobacco mosaic virus has been inactivated by many different reagents, some of which are known to modify specific protein groups (Stanley, 1940). In the case of inactivation by formaldehyde, it was shown that the inactivation was accompanied by abolition of amino groups and that removal of formaldehyde was accompanied by an increase in free amino groups and by

partial reversal of the inactivation (Ross and Stanley, 1938). Although other viruses have been inactivated by formaldehyde, the structural changes brought about by formaldehyde were not established (Stanley, 1940). The formaldehyde experiments are the only ones in which the inactivation of tobacco mosaic virus has been associated with definite changes in protein groups by chemical tests on the modified protein. In no case has it hitherto been shown that tobacco mosaic virus or any other virus can be modified structurally by chemical procedures *in vitro* and still produce disease.

In the study of enzymes several cases have been found in which the enzyme structure can be altered without inactivation of the enzyme. The amino groups of pepsin can be acetylated without loss of the proteolytic activity (Herriott, 1934). Carboxypeptidase is active even in the presence of formaldehyde (Anson, 1937). Some of the SH groups of urease can be oxidized without any change in the urease activity (Hellerman, 1939).

The inoculation of Turkish tobacco plants with virus whose SH groups have been abolished with iodine results in the production of virus with the normal SH content. If the virus in the inoculum is not reduced within the living plant cells to virus with a normal SH content it must be concluded that iodine-modified virus causes the production, not of exact replicas, but of normal or unmodified virus. Such a result might be expected if virus with a normal SH content represents the nearest structure to the modified virus which may be synthesized within the plant cells due perhaps to some preexisting pattern. If, however, the inoculation of modified virus is followed by its reduction within the living plant cells to virus with a normal SH content it would be expected that the latter would stimulate the production of more normal virus. At the present time it is not known which of these alternatives represents the true course of events. It was found, however, that iodine-treated virus is not reduced to normal SH virus by a mash of normal tobacco plants. Unfortunately this is not absolute proof that such reduction does not take place in the living plant cells. Nevertheless it seems likely that reduction to SH of groups oxidized beyond S-S does not occur within the cells and hence that the present experiments provide an example in which a virus has been altered structurally without perpetuation of the structural changes in subsequent generations. Although at the present time it is impossible to assign definite reasons for the failure to perpetuate the structural change the results are important in connection with any consideration of the mechanism of virus reproduction.

If enough iodine is added to egg albumin (Anson, 1941) or to tobacco mosaic virus, not only are the SH groups abolished but the tyrosine groups are converted into di-iodotyrosine groups. When enough iodine is added

to tobacco mosaic virus to iodinate the tyrosine groups, the virus is inactivated. This result does not of itself prove that the inactivation is due to the change in the tyrosine groups and not to some other iodine reaction which occurs under similar conditions. However, it is known that when enough iodine is added to insulin (Harrington and Neuberger, 1936) or pepsin (Herriott, 1937) to iodinate the tyrosine groups, these proteins are likewise inactivated.

Whether or not it is possible to convert some of the tyrosine groups of tobacco mosaic virus into monoiodo or di-iodotyrosine groups without inactivating the virus is not decided by the present experiments.

Iodoacetamide at pH 8.0 under the conditions used in the present experiments abolishes few if any SH groups of tobacco mosaic virus, but nevertheless almost completely inactivates the virus. This result is of some interest for iodoacetamide has been regarded as a specific reactant for protein SH groups. It is hoped that the nature of the reaction which results in the inactivation by iodoacetamide will be elucidated in future work.

The present experiments raise such questions as whether all viruses, like tobacco mosaic virus, have SH groups which react with iodine but not with porphyrindin; whether in all cases oxidation of the SH groups fails to cause irreversible inactivation or indeed any change in the general character of the disease or in the type of virus produced in the infected plant; and whether all viruses can be inactivated by concentrated iodine and iodoacetamide. The fact that a virus variant was not produced by changes such as those described in the present paper does not mean that the production of variants by chemical treatment is impossible. It may be that the production of chemical variants must await the development of techniques for changing the amino acid content or arrangement of a virus without causing loss of virus activity, rather than merely changing amino acid groups such as SH. Experiments of the kind which have been done with tobacco mosaic virus in which the changes in both protein structure and activity are followed can, in case of necessity, be carried out with only a few milligrams of purified virus. It is to be hoped that similar experiments will be carried out with different viruses and also that different reactions will be used in the attempt to produce virus variants *in vitro* by definite chemical changes of protein structure.

EXPERIMENTAL

The Nitroprusside Test.—The nitroprusside test used in the present experiments is carried out as previously described in a solution of guanidine hydrochloride prepared from purified guanidine carbonate (Anson, 1941).

With recrystallized egg albumin about the same pink color is obtained with nitroprusside, whether 1 drop of 0.1 M cyanide is added or not. This small amount of cyanide suffices to combine with heavy metal impurities and does not cause any significant reduction of S-S to SH. With some samples of tobacco mosaic virus, however, an extremely weak nitroprusside test is obtained unless a drop of dilute cyanide is added. This indicates that some samples of tobacco mosaic virus may contain impurities which interfere with the nitroprusside test for SH groups.

The cyanide-nitroprusside test for S-S groups which are reduced to SH by cyanide is conveniently carried out as previously described (Anson, 1941) by adding 1 drop of 2 N cyanide to the protein in strongly alkaline guanidine hydrochloride solution and adding the nitroprusside 5 minutes later. The nitroprusside test carried out with no cyanide or with dilute cyanide which does not reduce S-S will be referred to as the nitroprusside test. When strong cyanide which can reduce S-S is added, the test will be called the cyanide-nitroprusside test.

SH Titrations.—The SH groups of denatured egg albumin can be estimated by allowing the protein to stand 45 minutes in neutral guanidine hydrochloride solution and then determining how much porphyrindin must be added to abolish the nitroprusside test (Greenstein, 1938). This method was applied to tobacco mosaic virus, and it was found that 1 cc. of 0.0006 N porphyrindin was required for 10 mg. of virus in order to abolish the nitroprusside test (Stanley and Lauffer, 1939).

The SH titration in guanidine hydrochloride solution has recently been modified in two ways. First, ferricyanide, tetrathionate, and *p*-chloromercuribenzoate are used as titrating agents instead of porphyrindin. Second, the titrating agent is added before the guanidine hydrochloride instead of 45 minutes thereafter (Anson, 1941). When purified guanidine hydrochloride is used, the same SH titration value for egg albumin is obtained whether ferricyanide is added before or after the guanidine hydrochloride. When the guanidine hydrochloride happens to contain impurities —which almost all commercial samples tested were found to contain—then low results are obtained by the original procedure because some SH groups are oxidized while the protein is standing in guanidine hydrochloride solution before the addition of ferricyanide. When ferricyanide is added to tobacco mosaic virus 45 minutes after the guanidine hydrochloride, different results are obtained by titrating the SH groups of different samples of virus even when purified guanidine hydrochloride is used, because some samples of virus themselves contain impurities which bring about the abolition of SH groups in guanidine hydrochloride solution. When guanidine hydrochloride of suitable purity is used and the titrating agent is added before

the guanidine hydrochloride, then 1 cc. of 0.00056 N ferricyanide, tetrathionate, or mercuribenzoate is needed to abolish the nitroprusside test of 10 mg. of tobacco mosaic virus, and all samples of virus give the same titration value. In the following experiments, SH groups are estimated by the ferricyanide titration method with ferricyanide added before the guanidine hydrochloride according to the directions previously described (Anson, 1941).

The SH groups of egg albumin (Anson, unpublished results) and of tobacco mosaic virus can also be estimated by measuring the blue color obtained when the proteins reduce Folin's uric acid reagent in neutral urea solution. The values obtained agree with those obtained by the ferricyanide titration.

Ross (1940) found that tobacco mosaic virus contains no methionine and a total amount of SH plus S-S sulfur which, within the experimental error, accounts for the total sulfur content of the virus of 0.2 per cent. The total SH plus S-S was estimated by titrating the SH groups in an HI hydrolysate, in which any S-S has been reduced to SH. The present SH titrations in guanidine hydrochloride solution confirm earlier results (Stanley and Lauffer, 1939) and show that all the sulfur of tobacco mosaic virus can be accounted for by SH alone.

Reactions with Iodine.—Tobacco mosaic virus prepared by differential ultracentrifugation (Stanley and Wyckoff, 1937; Stanley, 1937) has been treated with iodine under various conditions and the products tested for SH groups, for groups which can be reduced to SH by cyanide, for tyrosine groups by the Millon test, and for virus activity. The results are given in Table I. In the first experiments small amounts of iodine are added to neutral tobacco mosaic virus at 0°C., all the iodine is consumed, and the minimum amount of iodine is found which abolishes the nitroprusside test in guanidine hydrochloride solution.

0.5 cc. of iodine solution (prepared by diluting a stock solution of 0.1 N I_2 in 0.18 N KI) is added to 0.5 cc. of 2 per cent tobacco mosaic virus plus 0.1 cc. of a buffer consisting of equal parts 1 M Na_2HPO_4 and 1 M NaH_2PO_4. All the solutions are precooled in ice water. After this solution has stood 30 minutes at 0°C. and the solution gives no color with starch, the nitroprusside test in guanidine hydrochloride solution is carried out. The test is positive if the amount of iodine added is 1.5 times the amount theoretically needed to oxidize the SH groups found in denatured tobacco mosaic virus to S-S, and negative if 2.5 times the theoretical amount is added. Even when no test for SH groups is obtained, a strong test is obtained if the iodine-treated protein is first exposed to alkaline cyanide which can reduce S-S to SH.

If iodine is added to native egg albumin at 0°C. and in the presence of 1 N KI, the SH groups of egg albumin can be abolished by the addition of

1 cc. of 0.001 N iodine to 10 mg. of native egg albumin. This is exactly the amount theoretically needed for the oxidation of the SH groups in denatured egg albumin to S-S. If 2 cc. of 0.001 N iodine is added, then 1 cc. of 0.001 N iodine is consumed, as shown by back titration with thiosulfate (Anson, unpublished results). When 1 cc. of 0.00056 N I_2 (the theoretical amount) is added to 10 mg. of neutral tobacco mosaic virus at 0°C. and in the presence of 1 M KI, no iodine is used up in 30 minutes, as shown by back titration with thiosulfate.

TABLE I

Reactions of Iodine with Tobacco Mosaic Virus

Composition of reaction mixture 0.5 cc. virus solution 0.5 cc. I_2 solution 0.1 cc. PO_4 solution		Temperature	Time	Nitroprusside test	Cyanide-nitroprusside test	Millon test	Lesions per half leaf		Estimated amount of inactivation*
Concentration of virus solution	Concentration of I_2 solution						Iodine-treated virus	Control virus	
mg. per cc.	*N*	°C.	*hrs.*						*per cent*
20	0.00168	0	0.5	−	+	+	56.5	65.5	14
20	0.00280	0	0.5	−	+	+	70.0	79.0	11
10	0.01	37	2	−	+	+	35.7	35.4	0
10	0.04	37	2	−	−	+	29.1	29.2	0
10	0.04	37	8	−	−	+	79.2	104.5	24
10	0.04	37	24	−	−	+	35.8	46.0	22
10	0.05	37	2	−	−	+	28.8	34.8	17
10	0.06	37	2	−	−	+	25.4	32.2	21
10	0.06	37	2	−	−	+	31.2	47.7	35
10	0.03	55	0.5	−	−	+	15.3	30.1	49
10	0.04	55	0.5	−	−	+	11.6	22.0	47
20	0.1	37	2	−	−	−	2.5	30.3	92
10	0.1	37	2	−	−	−	4.5	34.5	87
10	0.05	60	1	−	−	−	0.0	20.2	100

* Differences of less than about 20 per cent are not regarded as indicating a significant difference in virus activity.

One cannot decide on the basis of our experiments alone why the SH groups of tobacco mosaic virus, unlike the SH groups of free cysteine and of egg albumin, do not react with dilute iodine in 1 N KI. It may be that the SH groups of tobacco mosaic virus or their precursors are less reactive than the corresponding groups of egg albumin. It is also possible that there are spatial obstacles to the ready formation of S-S groups in native tobacco mosaic virus (*cf.* Neurath, 1940).

One might suppose that the tyrosine groups of native egg albumin would react with dilute iodine in 1 N KI, even if the SH groups fail to react. Even free tyrosine, however, does not react with dilute iodine if the solution contains 1 N KI (Anson, unpublished experiments).

In the second series of experiments, enough iodine is added to abolish

the cyanide-nitroprusside test but not the Millon test, which is positive for tyrosine groups and negative for di-iodotyrosine groups. All the iodine is not absorbed, so the excess iodine is removed before the tests are carried out. When 0.05 N I_2 is added under the conditions chosen, the cyanide-nitroprusside test is negative, the Millon test is strongly positive, indicating that few tyrosine groups have been converted into di-iodotyrosine groups, and the virus activity is essentially unaffected as shown by the fact that the iodine-treated virus produces about as many lesions as an equal amount of untreated virus. As the amount of iodine added or the temperature is increased, the Millon test and the activity become weaker and insoluble protein is formed. Similar results can be obtained by adding 0.05 N I_2, that is, without increasing the iodine concentration, if the reaction is carried out for a day instead of 2 hours or if the solution is made more alkaline.

The experiments are carried out as follows. To 0.5 cc. of 1 per cent virus there are added 0.1 cc. of 1 M phosphate buffer at pH 6.8 and 0.5 cc. of iodine solution. The resulting solution is kept at the designated temperature and period of time in glass stoppered weighing bottles, then 0.5 cc. of thiosulfate of the same concentration as the iodine is added, and finally the mixture is made up to 5 cc. with water. For the color tests the protein is precipitated with 0.2 N trichloracetic acid, centrifuged, stirred up with 0.2 N trichloracetic acid, and centrifuged again. For the activity measurements, the solution is diluted 10 times with 0.1 M phosphate buffer at pH 7.0 and the virus activity compared with that of an equal amount of control virus by the half-leaf local lesion method on 20 or more leaves of *Nicotiana glutinosa* (Loring, 1937). The control virus is kept under the same conditions in the absence of iodine and at the end of the reaction tetrathionate instead of thiosulfate is added. At the virus concentrations used there is a direct proportionality between the virus activity and the number of lesions produced on *Nicotiana glutinosa* leaves although differences less than about 20 per cent in the lesion count are usually not regarded as indicating a significant difference in virus activity (Loring, 1937).

Although HI is a strong reducing agent, cysteic acid (RSO_3H) is not reduced to SH by HI under the conditions of the Baernstein HI hydrolysis of proteins (Kassell, 1940). Even when 0.04 N iodine is used to oxidize the SH groups of tobacco mosaic virus beyond the S-S stage, as shown by a negative cyanide-nitroprusside test, the protein on being dialyzed and then hydrolyzed with HI still yields as much cysteine as protein not treated with iodine. The oxidized groups are still reduced to SH by HI. This shows that the SH groups oxidized by iodine beyond the S-S stage are not oxidized as far as RSO_3H. We are indebted to Dr. A. F. Ross for carrying out the HI hydrolysis and estimating the cysteine content of the HI hydrolysate.

In the final experiment, a 1 per cent solution of virus is treated with an equal volume of 0.05 N iodine at 60°C. for 1 hour. The cyanide-nitroprusside and the Millon tests are negative, and the virus is completely inactivated. By carrying out the reaction at 60°C. instead of at 37°C.,

one avoids the formation of insoluble protein. If the protein is insoluble, one cannot be sure that the inactivation is due to the chemical change and not to the insoluble state of the protein.

SH Groups of Virus Produced in Plants Infected with Iodine-Modified Virus.—The following experiments show that the inoculation of Turkish tobacco plants with virus whose SH groups have been abolished by iodine is followed by the production of virus with the normal number of SH groups.

For the first experiment Turkish tobacco plants were infected with the virus partially inactivated by treatment with 0.06 N iodine at 37°C. as already described. Virus was isolated from the plants after they had been infected for 6 weeks and the SH of the virus was titrated with ferricyanide. The first time this experiment was carried out, virus was obtained whose SH groups were abolished by half the amount of ferricyanide normally required. When this experiment was repeated several times, however, the virus isolated from plants infected with iodine-treated virus always gave the normal ferricyanide titration value. Furthermore, tobacco plants infected with the virus which gave the low titration value also yielded virus with the normal titration value. It is not known why in one case and in only one case virus with a low titration value was obtained. It is possible that this one sample of virus became accidentally contaminated with impurities which interfered with the estimation of the SH groups.

The possibility existed that the iodine-treated virus preparation used to infect Turkish tobacco plants for the production of more virus contained a very small amount of virus which had escaped reaction with iodine, that this normal virus multiplied in the Turkish tobacco plants much more rapidly than the modified virus, and that the normal virus finally obtained had its origin in the small amount of normal virus which had escaped reaction with iodine. In one series of experiments, therefore, the iodine-treated virus was used to infect not Turkish tobacco, in which tobacco mosaic virus causes a systemic infection, but *Nicotiana glutinosa*, in which tobacco mosaic virus causes local lesions. When sufficiently dilute virus is used to infect such plants, each lesion is believed to contain only a single strain of virus, a fact which makes possible the separation of different strains of virus (Jensen, 1933; Kunkel, 1934). Turkish tobacco plants were infected by means of inocula prepared from single lesions previously obtained by rubbing iodine-treated virus over the leaves of *N. glutinosa* plants. The virus isolated from these Turkish tobacco plants was found to have a normal SH content.

In the first experiment, tobacco mosaic virus was treated with an equal volume of 0.01 N iodine for 2 hours at 37°C. as in the previous experiment with 0.04 N iodine. The

resulting virus gave a negative nitroprusside test but a positive cyanide-nitroprusside test. When tested against an untreated sample at a dilution of 10^{-4} gm. per cc. by the half-leaf method, the iodine-treated preparation gave an average of 35.7 lesions per half leaf and the control an average of 35.4 lesions per half leaf. If the lesions produced by the inoculation of iodine-treated virus had been caused only by virus which had escaped reaction with iodine, the number of lesions would have been greatly reduced by the iodine treatment. The virus treated with 0.01 N iodine was then used at a dilution of 10^{-6} gm. per cc. to inoculate the entire area of four leaves of a *Nicotiana glutinosa* plant. Five discrete and well separated lesions were selected and each was removed, macerated, and used as an inoculum for a group of four Turkish tobacco plants.

In a second experiment seven groups of four Turkish tobacco plants were infected by means of inocula prepared from seven single lesions obtained from virus almost completely inactivated by 0.1 I_2 instead of from virus not inactivated at all by 0.01 N I_2. It is extremely unlikely that this preparation contained any virus which had not reacted with iodine.

To 20 cc. of 2 per cent tobacco mosaic virus there were added 4 cc. of 1 M phosphate buffer at pH 7.4 and 20 cc. of 0.1 N iodine in 0.18 N potassium iodide. The mixture was kept at 37°C. for 2 hours, 20 cc. of 0.1 N thiosulfate was added, and the final solution was dialyzed overnight against cold distilled water in a shaking dialyzer. A precipitate of insoluble material was removed by centrifugation and found to contain 170 mg. of protein. The supernatant solution contained 0.2 mg. of virus per cc. which when applied to half leaves at a concentration of 10^{-4} gm. per cc. gave an average of only 2.5 lesions per half leaf. Since the untreated starting material when applied at a concentration of 10^{-4} gm. per cc. gave an average of 30.3 lesions per half leaf on the other halves of the same leaves the soluble fraction was about 90 per cent inactivated. The largely inactivated soluble fraction of the virus treated with 0.1 N iodine was used at a concentration of 10^{-4} gm. of protein per cc. to inoculate the entire area of four leaves of a *Nicotiana glutinosa* plant, in order to obtain the single lesions used to infect Turkish tobacco plants.

The character of the lesions produced by virus treated with 0.01 N or 0.1 N iodine and the course of the infection were the same as those observed when *Nicotiana glutinosa* plants are infected with untreated virus.

After 5 weeks the groups of Turkish tobacco plants infected from single lesions produced by virus treated with 0.01 N and 0.1 N iodine were cut and frozen as well as a group of Turkish tobacco plants inoculated at the same time with untreated virus. Several samples from each of the three groups were macerated and the virus isolated by the procedure involving differential centrifugation customarily employed in this laboratory. In all cases the virus finally isolated had the normal SH content as measured by ferricyanide titration and the normal specific activity as measured by the number of local lesions produced on half leaves.

The experiments which have been described show that the disease caused by virus whose SH groups have been abolished by iodine is not due to residual virus which escaped reaction with iodine. They show further that the iodine treatment does not produce a new variant. So far as one can tell by the tests used, iodine-treated virus brings about normal infection and the production of normal virus. The results do not permit a decision as to whether or not iodine-treated virus is reduced in the living plant to

normal virus before multiplication of virus takes place. It was proved, however, that a mash of Turkish tobacco plants does not reduce virus which has been oxidized by iodine.

The virus added to macerated Turkish tobacco leaves was treated with an equal volume of 0.04 N I_2 for 2 hours at 37°C., as previously described. After the addition of thiosulfate to destroy the excess iodine and dialysis, it gave a negative cyanide-nitroprusside test and a positive Millon test. 30 mg. of the iodine-treated, dialyzed virus was added to 20 gm. of a mash prepared by macerating by means of a meat grinder the fresh leaves of a normal Turkish tobacco plant. The mixture was allowed to stand overnight at room temperature and the juice was expressed and subjected to the purification process involving differential centrifugation customarily employed in this laboratory. The 18 mg. of virus which was isolated was found to give a negative cyanide-nitroprusside test.

TABLE II

Effect of Iodoacetamide on the Activity of Tobacco Mosaic Virus

Concentration of virus	Concentration of iodoacetamide	Temperature	Time	Lesions per half leaf		Estimated amount of inactivation
				Virus treated with iodoacetamide	Control virus	
mg. per cc.	*M*	*°C.*	*hrs.*			*per cent*
0.5	0.05	37	2	13.3	24.2	45
0.5	0.05	37	18	0.6	23.2	97
5	0.1	37	6	2.7	12.9	79
5	0.1	55	4	1.1	10	89

Reactions with Iodoacetamide.—Iodoacetamide at pH 9.0 abolishes 40 per cent of the SH groups of native egg albumin (Anson, 1940). Iodoacetamide (prepared according to Anson, 1939) was added to tobacco mosaic virus in 0.1 M phosphate buffer adjusted to pH 8 with NaOH, and the concentrations of the reagents and the time and temperature of the reaction were varied as shown in Table II. More alkaline solutions were not used in order to avoid inactivation of the virus by alkali. The virus in aliquot portions of the various preparations treated with iodoacetamide was precipitated and washed with trichloracetic acid, dissolved in neutral guanidine hydrochloride solution, and titrated with ferricyanide. In every case the titration value was the same, within 10 per cent, as that obtained from normal untreated virus. In all the cases the virus was partially inactivated by iodoacetamide. The exact degree of inactivation, as shown in Table II, depended on the exact conditions of the reaction and in one case was as high as 97 per cent.

In a report (Anson, 1940) of some preliminary experiments, it was stated

that tobacco mosaic virus could absorb iodine without being inactivated and that neither tobacco mosaic nor rabbit papilloma virus was inactivated by iodoacetamide. In the present experiments inactivation was brought about by the use of much more concentrated iodoacetamide.

Non-Inactivation by p-Chloromercuribenzoate.—*p*-Chloromercuribenzoate, an SH reagent introduced by Hellerman (1939), combines with the SH groups of denatured egg albumin and denatured tobacco mosaic virus. It combines with native egg albumin either not at all or very loosely (Anson, 1941). In the present investigation it was found that 0.1 per cent tobacco mosaic virus is not inactivated at room temperature in a neutral solution containing 0.001 N mercuribenzoate, an amount which would combine with all the SH groups of the virus if the virus were denatured.

SUMMARY

1. Denatured tobacco mosaic virus has a number of SH groups corresponding to its total sulfur content of 0.2 per cent. The SH groups were estimated by titration with ferricyanide, tetrathionate, and *p*-chloromercuribenzoate in guanidine hydrochloride solution and by reduction of the uric acid reagent in urea solution.

2. The SH groups of tobacco mosaic virus or their precursors can be abolished by reaction of the native form of the virus with iodine.

3. Tobacco mosaic virus whose SH groups have been oxidized beyond the S-S stage by iodine but whose tyrosine groups have not been converted into di-iodotyrosine groups still retains its normal biological activity as shown by the number of lesions it causes on *Nicotiana glutinosa* plants and by the characteristic disease produced in Turkish tobacco plants.

4. The inoculation of Turkish tobacco plants with active virus whose SH groups have been abolished by iodine results in the production of virus with the normal number of SH groups.

5. If enough iodine is added to tobacco mosaic virus or if the iodine reaction is carried out at a sufficiently high temperature, then the tyrosine groups are converted into di-iodotyrosine groups and the virus is inactivated.

6. Tobacco mosaic virus can be almost completely inactivated by iodoacetamide under conditions under which iodoacetamide reacts with few if any of the protein's SH groups.

7. Tobacco mosaic virus is not inactivated by dilute *p*-chloromercuribenzoate.

REFERENCES

Anson, M. L., 1937, *J. Gen. Physiol.*, **20**, 663.

Anson, M. L., 1939, *J. Gen. Physiol.*, **23**, 247.

Anson, M. L., 1940, *J. Gen. Physiol.*, **23**, 321.
Anson, M. L., 1941, *J. Gen. Physiol.*, **24**, 399.
Arnold, V., 1911, *Z. physiol. Chem.*, **70**, 300, 314.
Greenstein, J. P., 1938, *J. Biol. Chem.*, **125**, 501.
Harrington, C. R., and Neuberger, A., 1936, *Biochem. J.*, London, **30**, 810.
Heffter, A., 1907, *Chem. Z.*, **11**, 822.
Hellerman, L., 1939, Cold Spring Harbor symposia on quantitative biology, Cold Spring Harbor, Long Island Biological Association, **7**, 165.
Herriott, R. M., 1934, *J. Gen. Physiol.*, **18**, 35.
Herriott, R. M., 1937, *J. Gen. Physiol.*, **20**, 335.
Jensen, J. H., 1933, *Phytopathology*, **23**, 964.
Kassell, B., 1940, *J. Biol. Chem.*, **133**, 1.
Kuhn, R., and Desnuelle, P., 1938, *Z. physiol. Chem.*, **251**, 14.
Kunkel, L. O., 1934, *Phytopathology*, **24**, 13.
Loring, H. S., 1937, *J. Biol. Chem.*, **121**, 637.
Neurath, H., 1940, Cold Spring Harbor symposia on quantitative biology, Cold Spring Harbor, Long Island Biological Association, **8**, 80.
Ross, A. F., 1940, *J. Biol. Chem.*, **136**, 119.
Ross, A. F., and Stanley, W. M., 1938, *J. Gen. Physiol.*, **22**, 165.
Stanley, W. M., 1937, *J. Biol. Chem.*, **121**, 205.
Stanley, W. M., 1940, The biochemistry of viruses, in Luck, J. M., Annual review of biochemistry, Annual Reviews, Inc., Stanford University, **9**, 545.
Stanley, W. M., and Lauffer, M., 1939, *Science*, **89**, 345.
Stanley, W. M., and Wyckoff, R. W. G., 1937, *Science*, **85**, 181.

THE EFFECT OF SONIC VIBRATIONS ON PHAGE, PHAGE PRECURSOR, AND THE BACTERIAL SUBSTRATE*

By A. P. KRUEGER, B. B. BROWN, and E. J. SCRIBNER

(From the Department of Bacteriology, University of California, Berkeley)

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During recent years mechanical vibrations produced by sonic and ultrasonic oscillators have been used extensively for two types of investigation in the field of bacteriology. Chambers, Mudd, Flosdorf, and coworkers at the University of Pennsylvania, have employed sonic vibrations to rupture bacterial cells and liberate antigenic substances. They have obtained detectable quantities of immunologically active components which normally are very labile, as for example, the soluble material of *H. pertussis* responsible for absorption of phase I agglutinins (1), the *E. typhi* Vi antigen (2), and the phagocytosis-promoting factor of the Lancefield C substance (3).

Another application has been the denaturation of various biologically active proteins such as enzymes and viruses. Flosdorf and Chambers (4) studied the inactivating effect of a frequency of 8900 cycles per second on egg albumin and found that the denaturation produced was comparable to that obtained with other denaturing agents. Rivers, Smadel, and Chambers (5) exposed vaccinia virus to the same frequency; the elementary bodies were inactivated but did not undergo disruption. Hapwood, Salaman, and MacFarlane (6) used ultrasonic waves generated by a quartz crystal vibrating at a natural frequency of 550 kc. per second for the treatment of vaccinia virus but did not observe any inactivation. In the case of tobacco mosaic virus, however, Stanley (7) found that the 550 kc. per second frequency produced fairly rapid inactivation. While conflicting results have been reported with reference to inactivation and denaturation, this is probably due in large measure to the wide variations in intensity, frequency, and mode of application of the vibrations.

We wish to report here the results of experiments undertaken to compare the rates at which phage, phage precursor, and staphylococci are destroyed by sonic vibrations.

* The authors wish to express their thanks to the John and Mary R. Markle Foundation and to Mr. Oscar Johnson for their generous support.

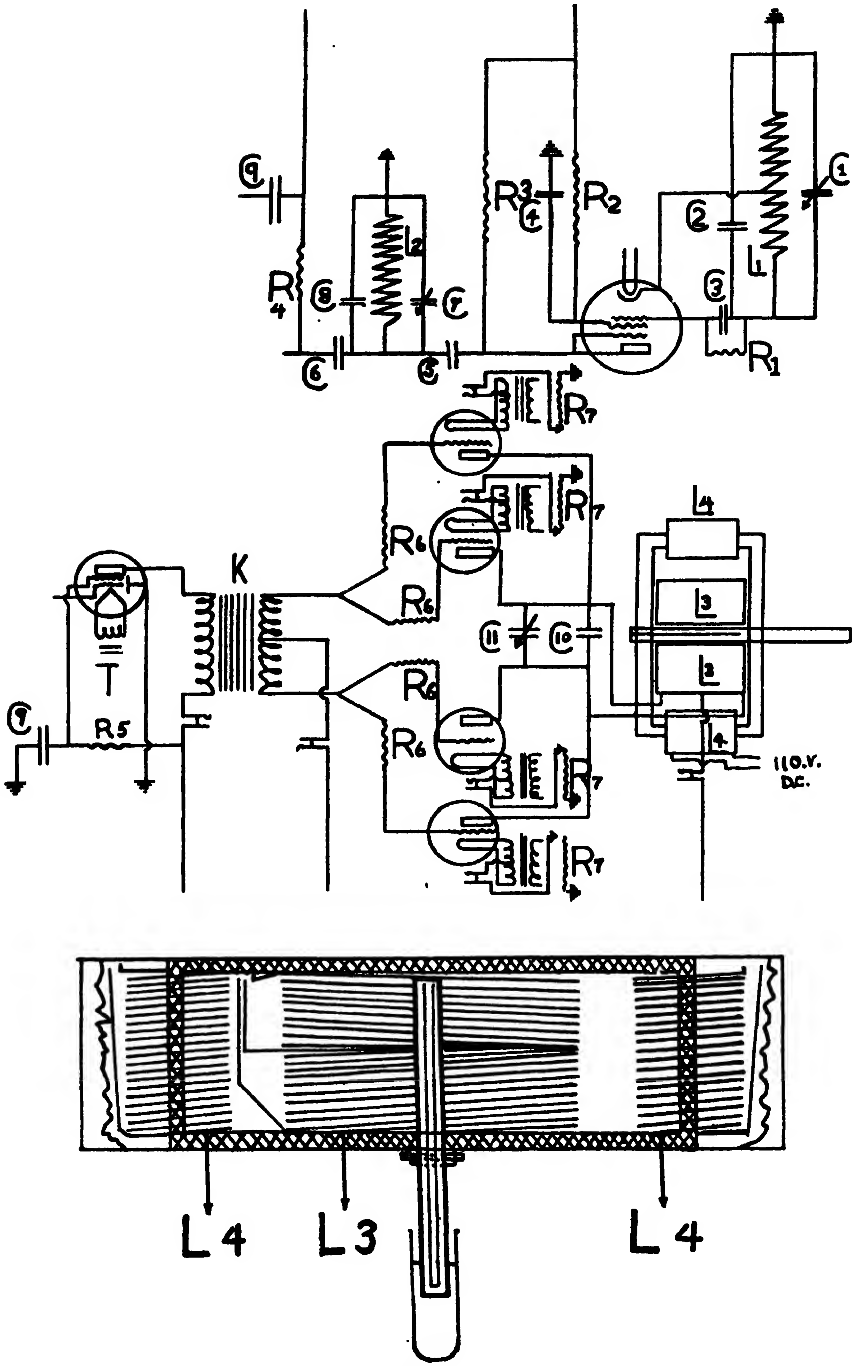
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FIG. 1

Methods

A magneto-striction oscillator of approximately 320 watts output was constructed to produce vibrations of 9300 cycles per second frequency in a 20 gauge nickel tube having an outside diameter of 13/16 inch. Fig. 1 gives the details of the circuit construction and the mounting of the nickel tube. In the oscillators previously reported the nickel tube has been pointed upward with the result that special provisions had to be made to maintain the material exposed in place over the tube. By constructing the tube so that it points downward it has been possible to circumvent many of the difficulties attending sonic treatment and we find that solutions to be studied can be placed in suitable open containers very conveniently. Two factors contributing to the operating efficiency of the oscillator are the improved tube suspension which diminishes damping to a great extent and the use of containers properly curved for focusing reflected sonic waves.

FIG. 1. Circuit diagram for sonic generator.

C-1	0.0005	µfd. air condenser	500 volt
C-2	0.001	" mica condenser	1,000 "
C-3	0.002	" " "	1,000 "
C-4	1.000	" " "	1,500 "
C-5	4.000	" paper "	750 "
C-6	1.000	" mica "	1,500 "
C-7	0.0015	" air "	1,000 "
C-8	0.0025	" mica "	2,500 "
C-9	0.01	" " "	2,500 "
C-10	0.009	" " "	7,500 "
C-11	0.00025	" castor oil condenser	12,500 "

L_1 = 2000 turns No. 22 gauge enameled copper wire layer wound 1 × 1½ inches. L_2 = 2000 turns No. 22 gauge enameled copper wire layer wound 1 × 1½ inches. L_3 = 12 series connected, stagger wound pancake coils of No. 22 gauge enameled copper wire, 123 turns each (1476 turns total) center tapped. These coils wound on 1½ inch core and separated by 10,000 volt insulating spacers. Complete assembled unit is 4 inches thick × 8 inches diameter. L_4 = 2 series connected, layer wound coils of No. 22 gauge enameled wire 250 turns each, cored with ½ inch × 2[illegible] *C* shaped mild steel pieces, gaped on ends at 0.020 inch. These coils pick up high voltages due to eddy currents so are insulated with 5,000 volt varnished cambric. Magnet is activated by 110 volts D.C. at 13.6 amps. K = a choke coil approximately ¾ Henry. The 4 lb. core is 2 square inches laminated high grade silicon steel 0.008 inch air gap. Primary 2 sections of 500 turns total No. 25 guage enameled copper wire with secondary interposed. Four leads. Secondary, 400 turns No. 23 guage enameled copper wire center tapped.

R_1 = 250,000 ohms resistance. R_2 = 100,000 ohms resistance. R_3 = 20,000 ohms – 2 watts. R_4 = 20,000 ohms – 2 watts. R_5 = 20,000 ohms – 2 watts. R_6 = 100 ohms – 2 watts. R_7 = 3,000 ohms – 75 watts.

The lower diagram shows the oscillating coil and D.C. magnets, as centrally located in a copper tank, 12 × 12 × 18 inches. Coal oil, chosen because of its high flash point and high dielectric constant is circulated at 6°C. through this tank and delivered under pressure to the bottom of the nickel tube. This device insures that the maximum temperature attained in the sample is not greater than 16°C.

Experiments were performed to determine whether the withdrawal of aliquots at intervals with consequent reduction in total volume of the exposed material would sufficiently alter energy absorption conditions to affect the rates of change. It was found that aliquot sampling restricted to small volumes did not detectably influence the slope of the curves for cell destruction or phage inactivation. For the sake of uniformity and convenience this sampling procedure was employed throughout our work rather than the more cumbersome method of exposing successive identical volumes for various time periods.

To study the rate of phage inactivation standard staphylococcus phage containing 1×10^{10} activity units/ml. was diluted to a titer of 1×10^{9} units/ml. in Locke's solution of pH 7.2. The solution was exposed to the action of the oscillator and samples were removed at intervals for determination of residual [phage] by the activity method (8). The rate of phage inactivation was found to serve satisfactorily as a measure of the oscillator's operating efficiency and the energy output was checked daily by running a phage inactivation experiment. Besides measuring the effect of the vibrations on phage alone we have carried out experiments on the rate of inactivation of phage in the presence of various concentrations of homologous staphylococci.

To determine the killing effect of the vibrations on staphylococci, suspensions containing approximately 5×10^{10} cells/ml. in Locke's solution at pH 7.2 were exposed and sampled at intervals, the residual viable cells being determined by plate counts.

For the study of phage precursor inactivation the following procedure was employed: 18 hour Roux flask cultures of staphylococci grown on nutrient agar were washed twice in Locke's solution and a broth suspension was prepared containing 5×10^{9} organisms/ml. The culture was maintained at 37°C. for 1 hour while oxygen was bubbled through the broth. At the end of this time an equal volume of broth was added and the oxygenation at 37°C. was continued for an additional hour. The cells were then centrifuged down and re-suspended in Locke's solution at a final concentration of 5×10^{10} cells/ml. Such "activated" cells when added to phage produce a tenfold increase in activity titer after only 2 minutes contact at 0°C. This phage-augmenting capacity has been attributed to the presence of phage precursor in the bacteria (9). The rate at which supersonic vibrations destroy intracellular precursor was followed by interval sampling. To a 4.0 ml. aliquot of each sample diluted to a bacterial density of 5×10^{8} cells/ml. was added 1.0 ml. of phage containing 1×10^{9} activity units/ml. The mixtures were kept at 5°C. for 5 minutes to allow the conversion of residual precursor into phage and were then titrated for phage activity. Plate counts were also made on the same samples to eliminate the possibility that any apparent reduction in precursor content might be ascribable to the killing of bacteria with subsequent irreversible sorption of phage.

EXPERIMENTAL RESULTS

When phage solution is exposed to the action of sonic vibrations of 9300 cycles per second frequency, there is no demonstrable lag period in the phage destruction curve; within 10 minutes the phage is reduced to about 0.1 per cent of the original titer (Fig. 2). In the cases of the precursor inactivation processand the lethal effect on bacteria there are lag phases of about 3 minutes and 30 minutes respectively (Fig. 3). It should be pointed out

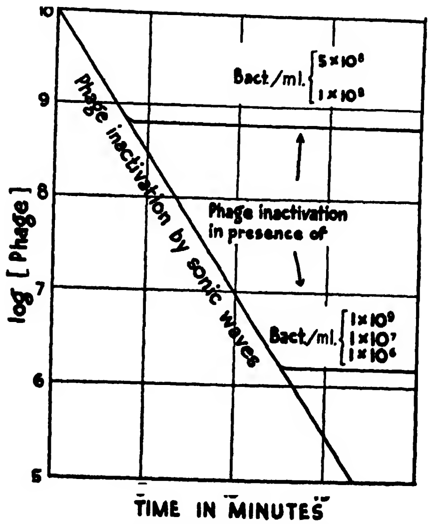

FIG. 2. Phage inactivation by sonic waves. The straight line represents the course of phage inactivation in the absence of bacteria. Phage inactivation in the presence of susceptible organisms follows the same curve for a time after which there is no further destruction of phage. The curve for phage alone is the average of twelve experiments and that for phage in the presence of bacteria is the average of ten experiments.

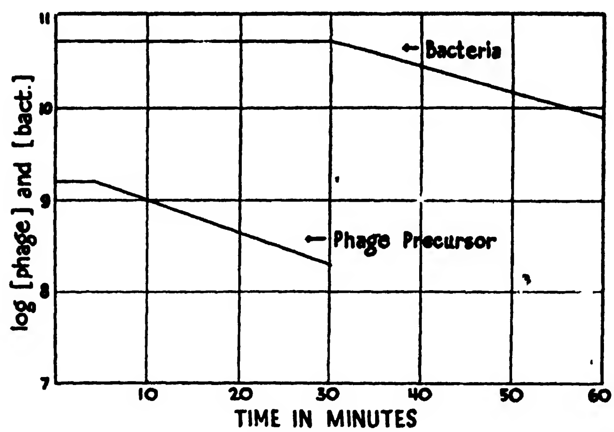

FIG. 3. The curves for inactivation of phage precursor and killing of bacteria by sonic waves. Residual intracellular precursor was measured by capacity of the cells to form phage when added to phage. Numbers of bacteria were determined by plate count. For both curves the data of eleven experiments were averaged.

that the curve for precursor inactivation is expressed as total phage produced when cells to be tested for precursor are added to a known amount of phage. The initial phage titer of the mixture is 2×10^8 activity units/ml. and any

increase above this level can be attributed to precursor reacting with phage to form more phage (10). The precursor inactivation experiments present a possible source of error in that the accumulation of any appreciable number of dead cells in the treated samples might bring about an apparent reduction in total phage formed without actually involving a direct loss of precursor. This depends upon the fact that dead cells can take up large amounts of phage; such sorption is irreversible and the attached phage does not participate in the titration (11). "Dead cells" as used here connote staphylococci which have lost their power to reproduce and if this criterion is employed there can be no error in the precursor inactivation curves for all the precursor is lost by the time the reproductive capacity of the staphylococci is measurably reduced. However, there remains the further possibility that cells treated with sonic vibrations may develop the property of taking up phage irreversibly before the reproductive mechanism is damaged. Control experiments were run on this point, using both normal and activated bacteria, and it was found that irreversible sorption does not take place until the cells have lost their ability to divide.

Once the lag phases are ended the destruction of precursor and the killing of staphylococci proceed logarithmically with time, the rate of the former process being somewhat faster than that of the latter. Theoretically one would expect the precursor to be destroyed much more rapidly than the cells, for it seems to be a protein (12) and its probable particle size may be assumed to be of the order of magnitude of phage itself; *i.e.*, very much smaller than the bacterium. Therefore the energy required for denaturation of the precursor molecule should be much less than the amount needed to destroy the cells. Actually, however, two facts modify this hypothesis. In the first place the precursor is intracellular and as a consequence is protected by the cell substance which no doubt absorbs a major share of the sonic vibrations. Secondly, in our experiments we have not measured the rate of cellular disruption but rather the rate at which the cells lose their ability to reproduce. Minor alterations of only a fraction of the total cell substance may be responsible for this loss. There is then no direct significance to be attached to the similarity between the two rates of inactivation. The fact that the precursor content of the cell can be abolished before the reproductive mechanism is damaged, is compatible with recently reported observations; namely that heat (12), iodoacetic acid (13), and methylene blue + light (14) can accomplish the same result.

The vast difference in particle size between the phage (about 50 $\mu\mu$) and the staphylococci (1,000 $\mu\mu$) can be invoked to account for the wide

variation in energy requirements for the destructive reactions. When phage is exposed to sonic vibrations in the presence of homologous organisms the rate of inactivation coincides initially with that for phage alone but plateaus are soon reached beyond which no further destruction of phage appears to occur. With [bacteria]'s of 5 × 10^8/ml. or 1 × 10^8/ml. the plateau begins after 4 to 5 minutes of exposure and the [phage] remaining is about 6 per cent of the original titer. Lower [bacteria]'s do not protect the phage as efficiently; with 1 × 10^7 and 1 × 10^6 bacteria/ml. the inactivation continues for 12 minutes and only 0.02 per cent of the original phage is left in active form. As in the case of precursor the inhibition of sonic inactivation probably depends upon absorption of energy by the staphylococcal cell substance. The organisms take up most of the phage and the intracellular phage fraction is protected by the relatively large volume of bacterial protoplasm around it. In general higher concentrations of bacteria confer a greater protective effect but for some unexplained reason the highest concentration of bacteria used, 1 × 10^9 cells/ml., gives no more protection than very low concentrations.

SUMMARY AND CONCLUSIONS

1. A nickel tube magneto-striction oscillator of 320 watts output producing sonic vibrations of 9,300 cycles per second frequency is described. Certain structural innovations contribute to operating efficiency and permit more convenient exposure of test materials than in earlier types.

2. The rate of phage inactivation by sonic waves proceeds logarithmically with time and serves as a satisfactory measure of energy output during operation of the generator. The curve for phage inactivation taking place in the presence of homologous staphylococci follows that for phage alone but soon reaches a plateau after which no further loss of activity is noted. In general higher concentrations of bacteria more effectively inhibit phage destruction than do lower concentrations.

3. Cells that have attained a resting state after a preliminary phase of rapid growth normally have the capacity of inducing a very rapid and marked increase in [phage] when added to phage. This effect has been attributed to the presence of intracellular phage precursor. The store of phage precursor in activated cells is destroyed by sonic waves in about 30 minutes. The number of cells (plate count) shows no reduction until after the precursor is entirely inactivated.

4. Attempts to extract phage precursor from activated staphylococci by exposing the cells to sonic vibrations were unsuccessful.

BIBLIOGRAPHY

1. Flosdorf, E. W., Kimball, A. C., and Chambers, L. A., *Proc. Soc. Exp. Biol. and Med.*, 1939, **41**, 122.
   Flosdorf, E. W., and Kimball, A. C., *J. Immunol.*, 1940, **39**, 287.
2. Chambers, L. A., and Flosdorf, E. W., *Proc. Soc. Exp. Biol. and Med.*, 1936, **34**, 631.
3. Mudd, S., Pettit, H., Lackman, D., and Czarnetzky, E. J., *Am. J. Path.*, 1936, **12**, 746.
4. Flosdorf, E. W., and Chambers, L. A., *J. Immunol.*, 1935, **28**, 297.
5. Rivers, T. M., Smadel, J. E., and Chambers, L. A., *J. Exp. Med.*, 1937, **65**, 677.
6. Hapwood, F. L., Salaman, M. H., and MacFarlane, A. S., *Nature*, 1939, **144**, 377.
7. Stanley, W. M., *Science*, 1934, **80**, 339.
8. Krueger, A. P., *J. Gen. Physiol.*, 1929–30, **13**, 557.
9. Krueger, A. P., and Mundell, J. H., *Science*, 1938, **88**, 550.
10. Krueger, A. P., and Scribner, E. J., *J. Gen. Physiol.*, 1938–39, **22**, 699.
11. Krueger, A. P., *J. Gen. Physiol.*, 1931, **14**, 493.
12. Krueger, A. P., Mecracken, T., and Scribner, E. J., *Proc. Soc. Exp. Biol. and Med.*, 1939, **40**, 573.
13. Krueger, A. P., and Scribner, E. J., *Proc. Soc. Exp. Biol. and Med.*, 1940, **43**, 416.
14. Krueger, A. P., Scribner, E. J., and Mecracken, T., *J. Gen. Physiol.*, 1940, **23**, 705.

EFFECTS OF NITROBENZENE AND BENZENE ON VALONIA

BY W. J. V. OSTERHOUT

(*From the Laboratories of The Rockefeller Institute for Medical Research*)

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Nitrobenzene[1] is of special interest since, like certain living cells, it is able to discriminate electrically[2] between Na^+ and K^+.

This paper describes certain experiments in which it was applied to *Valonia*. Its effect on the P.D. is seen[3] in Fig. 1. At the start the cell in sea water had a negative[4] P.D. of 6 mv. When 0.013 M nitrobenzene in sea water was applied the P.D. after a short latent period changed in a positive direction. The curve fell and then rose very slowly: the rise is termed "recovery" for convenience, but this does not mean that the cell is returning to its normal state. A similar result is obtained with guaiacol and with hexylresorcinol and the cause may be the same in all these cases. With guaiacol[5] and with hexylresorcinol[6] recovery is usually complete or nearly so but in these experiments with nitrobenzene it was very slow and often incomplete.[7]

The latent period in Fig. 1 is much shorter than with guaiacol and hexylresorcinol. But in many cases it is longer than in Fig. 1 and it may last 45 seconds. With some cells the descent of the curve is much more rapid and may resemble that found with guaiacol. The time of recovery in nitrobenzene is very variable but in no case is less than 5 minutes.

Nitrobenzene lessens the potassium effect. With normal cells replace-

[1] $C_6H_5(NO_2)$.

[2] Osterhout, W. J. V., Some models of protoplasmic surfaces, in Cold Spring Harbor symposia on quantitative biology, Cold Spring Harbor, Long Island Biological Association, 1940, **8**, 51.

[3] The experiments were made on *Valonia macrophysa*, Kütz., using the technique described in former papers (Osterhout, W. J. V., *J. Gen. Physiol.*, 1936-37, **20**, 13; regarding the amplifier see Hill, S. E., and Osterhout, W. J. V., *J. Gen. Physiol.*, 1937–38, **21**, 541). The temperature varied between 20 and 25°C.

No evidence of injury was seen in these experiments.

[4] The P.D. is called negative when the positive current tends to flow from the external solution across the protoplasm to the sap.

[5] *Cf.* Osterhout, W. J. V., *J. Gen. Physiol.*, 1936–37, **20**, 13.

[6] Osterhout, W. J. V., *J. Gen. Physiol.*, 1940-41, **24**, 311.

[7] True recovery occurred when the cells were replaced in sea water.

ment of sea water by 0.6 M KCl changed the P.D. in a negative direction *by 20 to 54 mv. but when both solutions contained 0.013 M nitrobenzene* the change was reduced almost to zero.[8] When sea water was replaced by "0.27 M K sea water" (sea water in which 0.27 M NaCl had been replaced by KCl) the change of P.D. in a negative direction was from 9 to 16 mv. But there was little or no change when both solutions contained 0.013 M nitrobenzene.

Evidently nitrobenzene makes K^+ and Na^+ act more nearly alike. How does this come about?

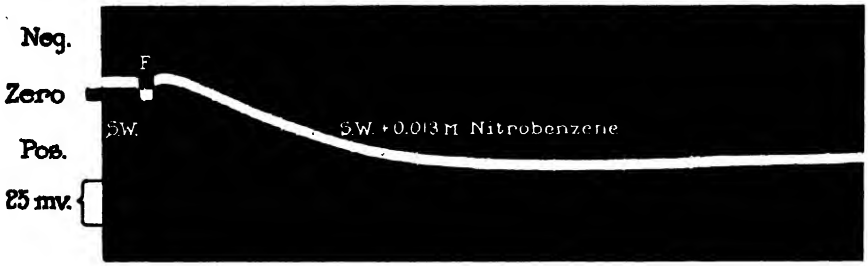

FIG. 1. At the start the cell had a negative P.D. of 6 mv. in sea water. When the cell was removed from the sea water the curve fell suddenly and registered the "free grid" (*F*) of the amplifier. Then nitrobenzene 0.013 M was added to the sea water and the curve jumped back to its former level and after a short latent period (including a slight rise) fell slowly until the P.D. was 41 mv. positive and later began to rise very gradually. Temperature 24°C. Time marks 15 seconds apart.

The behavior of Na^+ is changed as shown by the dilution effect. With normal cells dilution of the sea water to one half by isotonic glycerol (1.1M) containing 0.02 M $CaCl_2$ + 0.012 M KCl changed the P.D. in a negative direction to the extent of 5 to 12 mv. This change became less or disappeared entirely when the sea water contained 0.013 M nitrobenzene.

A similar result has been obtained with guaiacol[5] and with hexylresorcinol.[6]

This indicates that in normal cells u_{Na} is less than v_{Cl} but under the influence of the reagent this difference diminishes or disappears, making the behavior of Na^+ more like that of K^+ since normally we have $u_K > v_{Cl} > u_{Na}$.

Assuming that the partition coefficients (concentration in the non-aqueous

[8] The cells were first tested with 0.6 M KCl and returned to sea water. After a lapse of several hours they were tested with 0.6 M KCl + 0.013 M nitrobenzene. Failure to recover signifies an altered state of the cell (when dead the P. D. is zero).

protoplasmic surface ÷ concentration in the external solution) are equal[9] for KCl and NaCl it is evident that in order to abolish the potassium effect u_K must be equal to u_{Na}. Hence the loss of the potassium effect in the presence of the reagent indicates that $u_K = u_{Na} = v_{Cl}$. Since normally $u_K > v_{Cl} > u_{Na}$ this means that the reagent has increased u_{Na} and decreased u_K (assuming that v_{Cl} remains constant which is the simplest working hypothesis[10]).

It is of interest to note that when cells are in the "delayed polarization state"[11] they may be restored to the "regular polarization state" by application of nitrobenzene.

The changes in P.D. caused by nitrobenzene (Fig. 1) are antagonized to some extent by ammonia. After the positive change has occurred the addition of 0.002 M NH_4Cl at pH 8.1 usually changes the P.D. to some extent in a negative direction without necessarily bringing it back to the original value (before the nitrobenzene was added). The protoplasmic resistance[12] is increased by nitrobenzene but when ammonia is subsequently added it falls. Ammonia also tends to put the cells into the delayed polarization state.

The application of benzene (0.004 M to 0.008 M) produces effects resembling those described for nitrobenzene. Despite the differences between these substances[13] their effects are quite similar.

[9] The partition coefficients of KCl and of NaCl are assumed to be equal. This is done because we have no satisfactory way of estimating them. In order to make such an estimate we should need to measure the change of P.D. on diluting 0.6 M KCl (or a sea water rich in KCl) with an isotonic non-electrolyte but this is not practicable because in such solutions the P.D. constantly changes (*cf.* Damon, E. B., *J. Gen. Physiol.*, 1932 33, **16,** 375). Hence the apparent mobility as here used includes the partition coefficient in the sense that a higher partition coefficient of KCl would make u_K appear higher. It follows that the potassium effect becomes zero only when u_K equals u_{Na}.

[10] Since the resistance rises it is quite possible that v_{Cl} is not constant but it may still be true that the ratio $u_K \div v_{Cl}$ diminishes and the ratio $u_{Na} \div v_{Cl}$ increases which is all that the above discussion implies since when we say u_K and u_{Na} we really mean $u_K \div v_{Cl}$ and $u_{Na} \div v_{Cl}$, since v_{Cl} is always taken as unity. (*Cf.* Osterhout, W. J. V., *J. Gen. Physiol.*, 1929-30, **13,** 715.)

[11] Blinks, L. R., *J. Gen. Physiol.*, 1935 36, **19,** 633.

In this case the delayed polarization state was manifested by a lack of response or very little response to outward currents of less than 5 microamperes per square centimeter. No larger outward currents and no inward currents were applied since it seemed desirable to avoid changes in the surface due to current flow.

[12] The protoplasmic resistance was determined as described by Blinks and Skow (Blinks, L. R., and Skow, R. K., *J. Gen. Physiol.*, 1940 41, **24,** 247).

[13] The dielectric constants are, for benzene 2.2 (at 18°C.), for nitrobenzene 36 (at 20°C.). The latter is very polar as contrasted with benzene and is more soluble in water.

According to Blinks[14] acids raise the resistance in *Valonia* and this is antagonized by ammonia. He suggests (personal communication) that when reagents such as nitrobenzene and benzene produce a rise in resistance which is antagonized by ammonia the change may be due to production of acid by the cell which may also cause an alteration in P.D. (in a positive or negative direction, depending on the region where the principal production of acid occurs).

It may be noted, however, that the effects of nitrobenzene are not diminished by raising the pH of the solution to pH 9.5. The same changes in P.D. and resistance occur as at pH 8.1.

In conclusion it may be said that these results, together with those obtained with guaiacol[5] and hexylresorcinol,[15] make it clear that the behavior of inorganic ions can be greatly altered by organic substances. This important subject deserves further study.

SUMMARY

The effects of nitrobenzene and of benzene resemble those of guaiacol and of hexylresorcinol. The P.D. changes in a positive direction and then in a negative direction. The latter change may bring the P.D. back to the starting point with guaiacol and hexylresorcinol but with nitrobenzene and benzene this is not always the case.

The positive potential change produced by nitrobenzene and benzene may be antagonized to some extent by ammonia.

Nitrobenzene and benzene raise the electrical resistance and this is antagonized to some extent by ammonia.

The results afford a further illustration of the important fact that the behavior of inorganic ions can be changed by organic substances. The apparent mobility of Na^+ is increased and that of K^+ decreased by nitrobenzene and benzene (as is also the case with guaiacol and hexylresorcinol).

[14] Blinks, L. R., *J. Gen. Physiol.*, 1935-36, **19,** 655 *ff.* At low current densities the resistance is lessened or abolished by addition of ammonia and increased by addition of weak acids. Regarding *Halicystis* see Blinks, L. R., *J. Gen. Physiol.*, 1935-36, **19,** 867.

[15] Osterhout, W. J. V., *J. Gen. Physiol.*, 1939 40, **23,** 569.

since the data shown were deliberately selected to achieve this end. Moreover, nothing is known about the reproducibility of this particular set of results and, judging from comparisons made with other materials (3), one would not in general expect to obtain a similar coincidence with mercuric chloride if the tests were carried out under any other conditions.

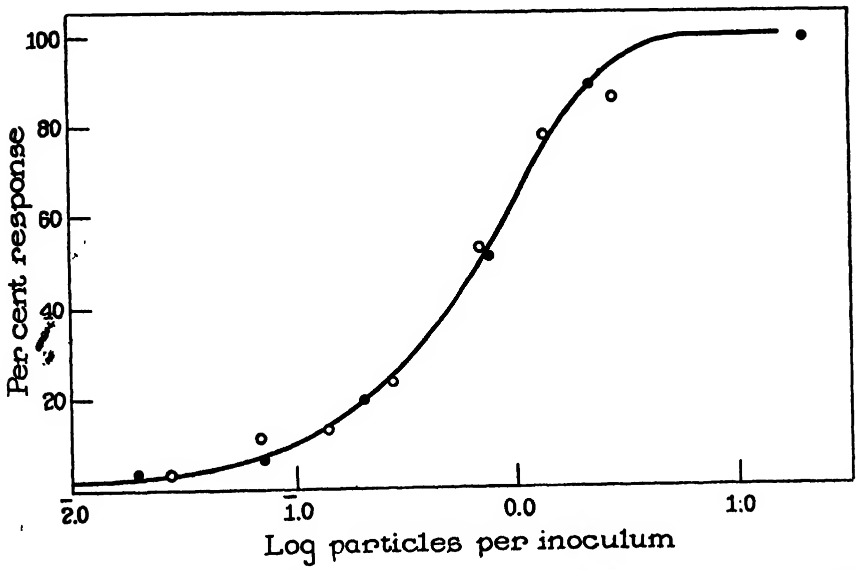

FIG. 1. Titration data obtained with vaccinia virus, mercuric chloride, and ideal infectious particles. The curve represents the response to single infectious particles predicted by Poisson's distribution:

$$\text{Average No. per inoculum} = -2.3 \log \frac{\text{negative inoculations}}{\text{total inoculations}}$$

The open circles are the data for the control animals in experiment 6 of Sprunt and McDearman (5) with vaccinia.

The closed circles are the data of Percival (4) for mercuric chloride in vaseline applied to the skin of thirty-five humans.

In either case the average number of particles per inoculum has been arbitrarily fixed at 0.69 for the 50 per cent response, reckoning the other doses proportional to concentration.

By contrast, recent observations with vaccinia reported by Sprunt and McDearman (5) have revealed a remarkable consistency in the behavior of this virus. They found that the resistance of rabbits could be increased by non-specific means so that the minimal reacting dose of virus was 30 to 50 per cent larger than for untreated rabbits. Nevertheless, the form of the titration curve obtained in treated and untreated rabbits was identical. Data from one of their experiments are included in Fig. 1. The deviation

from the one particle curve shown is systematic and might be attributed to a small degree of variation among the animals tested. On the other hand, if their data are to be interpreted as the coincidence of a geometric distribution of resistance having a standard deviation of approximately (log dose) 0.51, it seems remarkable that the operative procedures employed have affected the dispersion measure so little.[1]

In any case, it is apparent that the interpretation of the results of the titration of virus must rest for the present on the rather subjective criterion of inherent plausibility. On the basis of their own observations, Sprunt and McDearman (5) suggested, with some misgivings, that the effect of heightened resistance might be to decrease the fraction of virus particles capable of producing lesions, but that for either group of animals this fraction was constant and revealed itself in a one particle curve. Inasmuch as this conclusion seems at first sight contradictory, we are presenting some analogous results of our own with bacteriophage, of which the interpretation is clear.

It can be shown that the number of plaques produced on an agar plate, seeded with a given mixture of bacteriophage and bacteria, can be varied by changing certain conditions of bacterial growth, but that in all cases the individual plaques originate from the independent action of single particles of phage. In the experiment recorded in Table 1, two levels of "host resistance" were obtained by plating the mixtures of phage and bacteria in two different volumes of 0.7 per cent agar. Similar variations can be obtained by changing the concentration of agar (6), the nutrient composition of the medium, or the strain of bacterium employed. In the experiment shown here, the observed counts obtained with different aliquots of phage are in excellent agreement with the expected counts if each plaque results from one particle, whereas if even two particles were necessary, very different results would be obtained. The agreement is equally good for the two media, in spite of the twofold differences in the actual count. It is unlikely that any ambiguity is concealed by this agreement.

The finding with bacteriophage can be stated as follows: the probability that a given phage particle will produce a plaque varies with the conditions imposed, but under given conditions this probability is constant and inde-

[1] Actually, there may have been some effect not revealed by the chi^2 test employed by the authors. The average maximal per cent deviation from the theoretical curve for the six groups of untreated animals is 10.1 ± 1.6; for a similar number of treated animals it is 19.0 ± 3.4. All occur in the same region of the curve as illustrated in Fig. 1. The ratio of this difference to its standard error is 2.5, corresponding to 80:1 odds against obtaining this difference by accident.

pendent of any association between particles. It follows that no *a priori* objection can be made to the seemingly paradoxical interpretation suggested by Sprunt and McDearman of their results with vaccinia. The analogy with the bacteriophage is not, of course, to be construed as a confirmation of their findings.

Our own conclusion has been stated above in such a way as deliberately to avoid the fundamental question which arises concerning results of this kind. Do those particles which succeed in producing a plaque under given conditions do so because they possess some property distinguishing them from the remainder of the particles, or only because the success or failure of all the particles is determined by numerous local influences which fluctuate in a random manner? This question is irrelevant to the purely statistical interpretation of the data, but it suggests the possibility of attacking the more interesting problem of heterogeneity within the virus population itself.

REFERENCES

1. Parker, R. F., *J. Exp. Med.*, 1938, **67**, 725.
2. Bryan, W. R., and Beard, J. W., *J. Infect. Dis.*, 1940, **67**, 5.
3. Gaddum, J. H., Methods of biological assay depending on a quantal response, *Great Britain Med. Research Council, Special Rep. Series, No. 183*, 1933.
4. Percival, C. P., cited by Clark, A. J., The mode of action of drugs on cells, Baltimore, Williams & Wilkins Company, 1933, 107.
5. Sprunt, D. H., and McDearman, S., *J. Immunol.*, 1940, **38**, 81.
6. Bronfenbrenner, J., and Korb, C., *J. Exp. Med.*, 1925, **42**, 483.

SULFHYDRYL GROUPS OF EGG ALBUMIN IN DIFFERENT DENATURING AGENTS

By A. E. MIRSKY

(*From the Hospital of The Rockefeller Institute for Medical Research*)

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When egg albumin is denatured there occurs a striking change in its SH groups. This change provides a clue to an understanding of the change in configuration of the egg albumin molecule that takes place during denaturation. A protein can be denatured by many different agents. This is a well known and important characteristic of protein denaturation. In the present investigation our purpose is to discover whether different denaturing agents liberate the same or different numbers of SH groups in egg albumin.

The change in SH groups is readily observed. Native egg albumin does not give a color reaction with nitroprusside and does not reduce ferricyanide; denatured egg albumin gives the color with nitroprusside characteristic of SH groups and immediately reduces ferricyanide (4, 17). This change in the SH groups of egg albumin is an example of the general rule that certain groups in a protein become reactive as a result of denaturation. Other groups, in addition to sulfhydryl, become reactive when egg albumin is denatured. Some of these groups also reduce ferricyanide, though they require a more alkaline medium for their activity than do SH groups. These reducing groups are probably the phenolic groups of tyrosine (20). The SH groups of denatured egg albumin react with iodoacetate (17). Other groups of denatured albumin react with iodoacetate (22). Some of these groups, as yet unidentified, do not react with iodoacetate while the protein is native. There are proteins (in striated muscle and the crystalline lens of the eye) in which, unlike egg albumin, some SH groups are reactive even while the proteins are in the native state. Even while native, these proteins give a color reaction with nitroprusside and reduce ferricyanide (19). In these proteins denaturation produces a marked increase in the number of reactive SH groups. In other proteins (the serum proteins of the horse, for example) no SH groups are detectable either before or after denaturation.[1] But in both native and denatured serum proteins disulfide (S—S)

[1] Greenstein found that horse serum albumin gives a nitroprusside reaction in presence of a high concentration of guanidine hydrochloride (10, 11). I can confirm this observa-

groups can be shown to be present and they can be estimated after being reduced to SH groups (18). It is then found that there is a larger number of reactive S—S groups in the denatured than in the native serum proteins. The method used for estimating S—S groups in the serum proteins was subsequently used to estimate S—S groups in insulin (23) and lactalbumin (12). Of all the instances of reactive groups appearing in proteins after denaturation the occurrence of reactive SH groups in egg albumin is an example that presents several advantages for investigation: SH groups can be estimated with precision, and the complete absence of reactive groups in native egg albumin makes the increase in number on denaturation especially striking.

The present investigation deals with the effects of three different denaturing agents on the SH groups of egg albumin. The three denaturing agents are urea, guanidine hydrochloride, and the synthetic detergent, Duponol P. C. (a mixture of the C_{10}-C_{18} compounds of the series $CH_3(CH_2)n\ CH_2OSO_3$ Na) (1, 3, 8, 21). In each instance the denatured protein remains in solution while the denaturing agent is present.

Method

Protein SH groups are estimated by means of their reaction with ferricyanide, as a result of which they are oxidized to S—S groups and ferrocyanide is formed.

2 Protein SH + 2 ferricyanide = Protein S—S + 2 ferrocyanide

An excess of ferricyanide is added and the quantity of ferrocyanide formed is estimated. This is done by adding ferric sulfate which reacts with ferrocyanide to form Prussian blue which is estimated with a photoelectric colorimeter of the Evelyn type. The intensity of the blue color is a measure of the number of active, protein SH groups. Before adding ferric sulfate it is necessary either to remove the protein or to add some reagent that will keep protein in solution even in presence of ferric sulfate. Both procedures are followed.

For relatively simple SH compounds, such as cysteine and glutathione, the reaction with ferricyanide proceeds stoichiometrically. There is no difficulty in titrating the SH groups of glutathione with ferricyanide (16). The titration is simple and accurate, with a sharp end-point. Ferricyanide

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tion; a definite but not intense reaction is obtained. Estimation of the number of SH groups shows that less than 0.1 per cent is present, hardly a significant quantity.

has already been used to estimate the SH groups of a protein-denatured globin (20). It was at that time observed that "Whereas the oxidation of SH to S—S by ferricyanide is a definite reaction under suitable conditions (2 SH + 2 ferricyanide = 1 S—S + 2 ferrocyanide) the reaction of the other (reducing) groups (of a protein) with ferricyanide is not so definite. The greater the ferricyanide concentration and the longer the time of reaction, the more oxidation by ferricyanide takes place." At that time ferricyanide was not used to estimate the SH groups of denatured egg albumin. A more cumbersome procedure was followed. Conditions under which the reaction between ferricyanide and the SH groups of denatured egg albumin is precise and definite have now been found; the protein should be dissolved in approximately neutral solutions of urea, guanidine hydrochloride, or Duponol. Under these conditions the reaction goes with great speed. It is completed in less than 1 minute; no more ferricyanide is reduced in 60 minutes than in 1 minute. Nor within wide limits do the concentration of ferricyanide or temperature affect the quantity of ferricyanide reduced. These observations suggest that in the reaction between ferricyanide and egg albumin in neutral solutions of urea, guanidine hydrochloride, or Duponol only the SH groups of the albumin reduce ferricyanide.[2]

There are, as mentioned above, other reducing groups in denatured egg albumin, but those that have been investigated reduce ferricyanide in a slightly alkaline medium only. Furthermore, the reaction of these non-SH groups with ferricyanide is sluggish, there being no definite end-point, and the quantity of ferricyanide reduced depends upon the concentration of ferricyanide present. These groups, then, do not take part in the clearly defined reaction between egg albumin and ferricyanide in a neutral medium.

That the groups of denatured egg albumin which reduce ferricyanide in neutral medium appear to be SH groups and nothing but SH groups can be shown by using the nitroprusside test, especially in conjunction with certain reagents that combine with SH groups. This test in a protein may be considered to be specific for SH groups, for no other groups in a protein are known to give a color reaction with nitroprusside. It is possible that there

[2] After most of the experiments on egg albumin in solutions of urea and guanidine described in this paper were completed, Anson discovered the effect of Duponol (1). It was then that Duponol was used in the experiments reported in this paper. Anson found that the amount of ferricyanide reduced by denatured egg albumin in Duponol P C solution is within wide limits independent of the concentration of ferricyanide and the time, temperature, and pH of the reaction. The similar observations on egg albumin in solutions of urea and guanidine hydrochloride described in this paper had already been independently made.

are SH groups which do not give a nitroprusside test and which might react with some reagent other than ferricyanide. A detailed comparison under many different conditions of the color reaction of egg albumin with nitroprusside and the reducing reaction with ferricyanide shows a close correlation between these two reactions. When egg albumin reduces ferricyanide in neutral medium it gives a color with nitroprusside, and when it does not reduce ferricyanide it fails to give a color with nitroprusside. A number of examples of this correlation may be cited:

1. Native egg albumin does not give a color test with nitroprusside; nor does it reduce ferricyanide.

2. When egg albumin is denatured by urea, guanidine hydrochloride, Duponol, or any other agent, it gives a nitroprusside test and also reduces ferricyanide. After the reaction with ferricyanide is completed the albumin no longer gives a nitroprusside test.

3. Heat coagulated egg albumin is treated with ferricyanide and the excess ferricyanide is washed away when the reaction appears to be ended. The albumin no longer gives a test with nitroprusside. Guanidine hydrochloride is then added to the albumin. The albumin now gives a color reaction with nitroprusside and also reduces ferricyanide.

4. Egg albumin denatured by urea is oxidized with ferricyanide and the excess ferricyanide is removed. The albumin no longer gives a test with nitroprusside. The albumin is now treated with guanidine hydrochloride. Neither a nitroprusside test nor reducing action with ferricyanide is observed.

5. When guanidine hydrochloride is added to egg albumin solutions in the pH range from 5.8 to 7.8 precisely the same quantities of ferricyanide are reduced and in no case is a nitroprusside reaction observed when the excess ferricyanide is removed. When guanidine hydrochloride is added to albumin at pH 4.4, 21 per cent less ferricyanide is reduced. Now when the excess ferricyanide is removed and the albumin is tested with nitroprusside in presence of guanidine hydrochloride a slight color reaction is observed. This albumin, brought to pH 7.0, reduces ferricyanide in presence of guanidine hydrochloride. It reduces 16 per cent of the quantity it would have reduced if it had not previously reacted with ferricyanide at pH 4.4.

6. The SH groups of denatured egg albumin react with iodoacetate and iodoacetamide. Egg albumin, so treated, no longer gives a nitroprusside test; nor does it reduce ferricyanide.

7. SH groups of denatured egg albumin, like other SH groups, react with mercuric chloride. After the reaction the albumin neither gives a nitroprusside test nor reduces ferricyanide.

The close correlation between the nitroprusside test and the tendency to reduce ferricyanide makes it unlikely that there are any other groups in egg albumin in addition to SH which reduce ferricyanide in a neutral medium. It is also unlikely, quite apart from the nitroprusside test, that there are any groups other than sulfhydryl in egg albumin which combine with iodoacetate and mercuric chloride and which also reduce ferricyanide in neutral medium. And yet it should be recognized that the existence of such groups has not been *completely* excluded. Ferricyanide certainly reacts with all SH groups giving a nitroprusside test. But it is possible that there are a few non-SH groups in a protein that react with ferricyanide in neutral medium. In a protein the reactive range of other reducing groups may overlap to a slight extent with the range of activity of SH groups. With this reservation the quantity of ferrocyanide formed can be taken as a measure of the number of reacting SH groups. It remains to be shown that none of the ferrocyanide formed in the reaction between protein and ferricyanide is lost in those cases in which the protein is removed before estimating ferrocyanide. And, in fact, none is lost, for it is found that ferrocyanide added before removing protein is completely recovered when the protein is subsequently removed.

Denaturation

A protein is said to be denatured when it is insoluble in a medium in which it is soluble while still native. Egg albumin denatured by heat is insoluble in water at the isoelectric point, pH 4.7—a medium in which native egg albumin is soluble. The egg albumin in urea, guanidine hydrochloride, or Duponol, which reduces ferricyanide is denatured. It is soluble at the isoelectric point in the presence of urea, guanidine hydrochloride, or Duponol, but when these denaturing agents are removed or diluted with water the protein is found to be insoluble.

Experiments on egg albumin in urea solutions show clearly that liberation of SH groups and formation of insoluble protein are integral parts of the same process. To liberate the maximum number of SH groups 1 gram of urea is added to each 1 cc. of albumin solution. After standing for 60 minutes the albumin reduces no more ferricyanide than it does after standing for only 30 minutes; and within 30 minutes all of the albumin is denatured. This can be shown by diluting the urea solution with water, adjusting the pH to 4.7, and adding one quarter of the volume of saturated $(NH_4)_2SO_4$ (much less than is needed to precipitate native egg albumin). No protein is left in solution. The correlation between liberation of SH

groups and formation of insoluble protein becomes more apparent when insufficient urea is added to liberate all the SH groups. If the number of SH groups liberated is estimated at different intervals of time after adding urea, it is found that with advancing time more and more groups are liberated so that even after 3 hours in urea and ferricyanide no end-point is reached. Tests for presence of insoluble egg albumin (made by adding water, pH 4.7 acetate buffer, and saturated $(NH_4)_2SO_4$) show that part of the albumin is insoluble, but that some remains soluble. The soluble and insoluble fractions are separated from each other and both are washed free of ferricyanide. In the insoluble fraction there are no more SH groups; this fraction does not give a reaction with nitroprusside even in presence of guanidine hydrochloride. The soluble fraction still contains SH groups; if this albumin is denatured by adding guanidine hydrochloride an intense nitroprusside reaction is observed. Estimation of the number of SH groups in the albumin of the soluble fraction after denaturation by addition of Duponol shows that per milligram of protein there is the same number of groups as in egg albumin not previously treated with urea and ferricyanide. That fraction of egg albumin in urea and ferricyanide which becomes insoluble (when tested under certain clearly defined conditions) has all of its SH groups liberated, whereas albumin that still is soluble has none of its SH groups liberated. Denaturation of egg albumin by urea is a discontinuous process. A given molecule of protein is either native or denatured. Denaturation of egg albumin by some other agents, it will be shown in other papers, is also an all-or-none process.

When enough guanidine hydrochloride is added to egg albumin to liberate the maximum number of SH groups all of the albumin loses its solubility, when tested in the same manner as in the experiments with urea. In experiments with Duponol a different procedure is followed to demonstrate the altered solubility of albumin. The solution of albumin in Duponol is dialyzed against water for a long time to remove as much Duponol as possible. To the clear dialysate is added 1/10 its volume of saturated ammonium sulfate. This suffices to precipitate all the protein in solution, indicating that Duponol, as well as urea and guanidine, alters the solubility of egg albumin.

RESULTS

Since the only amino acid known to have SH groups is cysteine, the SH groups of egg albumin are considered to be part of cysteine, and are accordingly expressed as percentage of cysteine. The results are reproducible to within ±5 per cent.

It can be seen from Table I that the quantities of ferricyanide reduced by egg albumin in urea, guanidine hydrochloride, and Duponol are about

TABLE I

SH Groups of Egg Albumin Expressed As Per Cent of Cysteine, Denatured by Urea, Guanidine Hydrochloride, and Duponol

Albumin preparation No.	Albumin solution	Albumin	Ferricyanide added	Volume after adding buffer ferricyanide	pH	Denaturing agent added	Temperature	Time of reaction	Quantity ferrocyanide formed	SH groups	Cysteine
	cc.	*mg.*	*mM*	*cc.*		*mg.*	*°C.*	*min.*	*mM*	*mg.*	*per cent*
						(Urea)				(Cysteine)	
I	0.25	18.5	0.005	0.350	6.7	350	37.5	30	0.00145	17.5	0.95
							37.5	15	0.00144	17.3	0.94
							25.0	30	0.00144	17.3	0.94
							25.0	30	0.00140	16.8	0.91*
						(Guanidine hydrochloride)					
I	0.25	18.5	0.005	0.350	6.7	300	25.0	30	0.00142	17.0	0.92
			0.025						0.00144	17.3	0.94
			0.005					15	0.00144	17.3	0.94
			0.005				0	15	0.00145	17.5	0.95
			0.010		7.8		25.0	30	0.00146	17.6	0.95
			0.010		5.8		25.0	30	0.00143	17.2	0.93
			0.010		4.4		25.0		0.00114	13.7	0.74
						(Duponol)					
I	0.25	18.5	0.005	1.85	6.7	50	37.5	10	0.00153	18.35	0.99
	0.25	18.5	0.005	1.85	6.7		37.5	10	0.00148	17.8	0.96
II	0.20	20.8	0.005	1.85	6.7		37.5	10	0.00171	20.6	0.99
	0.20	20.8	0.005	1.85	6.7		37.5	10	0.00168	20.2	0.97
III	0.35	17.5	0.005	1.85	6.7		37.5	10	0.00137	16.45	0.94
	0.35	17.5	0.005	1.85	6.7		37.5	10	0.00143	17.15	0.98

* In this experiment the albumin was precipitated with tungstic acid before estimation of ferrocyanide. In the other experiments with urea, Duponol was added to prevent precipitation of protein during the ferrocyanide estimation.

the same. Guanidine hydrochloride and Duponol do not liberate any SH groups not liberated by urea, as the following experiment shows: Egg albumin in urea is oxidized by ferricyanide. After the urea and ferricyanide have been washed away, the albumin fails to give a reaction with nitroprusside or to reduce ferricyanide when placed in either guanidine hydrochloride or Duponol.

Three different preparations of crystalline egg albumin were used in the experiments described in this paper. For most of the experiments a single preparation was used. Two other preparations were made to learn whether different samples of egg albumin contain the same number of SH groups when denatured. Of these preparations one (preparation number II of Table I) was made by the method of Kekwick and Cannan (13) and the other (preparation number III of Table I) by La Rosa's method. Preparation III was investigated in the electrophoresis apparatus of Tiselius by Dr. Longsworth. No appreciable quantity of any protein constituent of egg white except egg albumin could be detected. All three preparations of albumin when denatured by Duponol were found to contain the same number of SH groups.

There have been several investigations of the SH groups of egg albumin denatured by urea and guanidine hydrochloride. Rosner estimated SH groups by means of their reaction with iodoacetate (22). He found 0.87 per cent in egg albumin denatured by urea. This is about 10 per cent lower than the result obtained by the reaction with ferricyanide—an entirely different method. Greenstein estimated SH groups by titrating them with porphyrindin, a powerful oxidizing agent (8). For egg albumin in urea he obtained 1.00 per cent SH and for albumin in guanidine hydrochloride 1.28 per cent. The results given by the porphyrindin and ferricyanide methods for the SH groups of albumin in urea are in good agreement. In guanidine hydrochloride the two methods do not agree; in this medium porphyrindin titration gives a much higher value. What seems especially significant in the porphyrindin titrations, and this point has been emphasized by Greenstein, is that different denaturing agents, such as urea and guanidine hydrochloride, liberate different numbers of SH groups. This apparent difference in SH groups seems to be due to a defect in method; porphyrindin may react with reducing groups other than SH in a denatured protein. This possibility was recognized by Kuhn and Desnuelle who first used porphyrindin for titrating protein SH groups (14). They accordingly carried out the reaction at 0° and in an especially careful manner. They placed some confidence in their results on heat coagulated egg albumin because they were in agreement with results obtained by other methods (17, 24). To Greenstein it seemed "hardly probable that the dye (porphyrindin) would react with other types of reducing groups in the protein. Such groups, involving tyrosine and tryptophane radicals, as Mirsky and Anson point out, only begin to make their presence felt at pH 10 and, moreover, react very slowly with ferricyanide and not at all with cystine or phosphotungstate. It is certain in any case that they would not exhibit

a nitroprusside reaction." The fact that the non-SH reducing groups of denatured egg albumin do not reduce ferricyanide at pH 7.0 but require a more alkaline medium does not mean that these groups will fail to reduce porphyrindin, a more powerful oxidant than ferricyanide, at pH 7.0 (and in the presence of guanidine hydrochloride). Indeed Kuhn and Desnuelle point out that porphyrexid (closely related to porphyrindin) oxidizes thiamin to thiochrome in neutral solution while potassium ferricyanide requires an alkaline solution for the same oxidation.[3] And although it may be certain that the non-SH reducing groups of denatured egg albumin "would not exhibit a nitroprusside reaction" this does not prove that they do not react with porphyrindin in presence of guanidine hydrochloride. Greenstein showed that the groups of egg albumin exhibiting a nitroprusside reaction reduce porphyrindin; but he did not show that the groups not exhibiting a nitroprusside reaction do not reduce porphyrindin. Denatured excelsin, he observed, neither gives a nitroprusside reaction nor reduces porphyrindin (9). On the other hand crystalline papain reduces more porphyrindin than can be accounted for by its sulfur content (5). There is then some doubt concerning the estimation of protein SH groups by titration with porphyrindin.[4] The difference in the quantities of porphyrindin reduced by egg albumin in urea and in guanidine hydrochloride (a difference of 28 per cent) is not due to there being an increased liberation of SH groups in guanidine hydrochloride, for if this were so, egg albumin in urea that had been oxidized with ferricyanide would subsequently give a nitroprusside reaction when dissolved in guanidine hydrochloride—and, as stated above, a nitroprusside test is not obtained under these conditions.

Anson finds the same number of SH groups (equivalent to a cysteine content of 1.2 per cent) present in egg albumin denatured by guanidine hydrochloride and Duponol (2).

To explain why active SH groups appear in egg albumin when it is denatured, measurements of SH groups must be combined with other kinds of information about the protein. Such investigations have already been carried out and will be described in another paper. The significance of the measurements made in the present investigation will then become clear, as will also the conclusion that the same number of active SH groups is present in egg albumin denatured by urea, guanidine hydrochloride, or Duponol.

[3] "Mit Porphyrexid lässt sich Aneurin in neutraler Lösung zu Thiochrom oxydieren, was sonst nur noch mit Kaliumferricyanid in alkalischer Lösung gelingt."

[4] The validity of SH estimations in proteins by titration with porphyrindin has also been questioned by Brand and Kassell (6).

SUMMARY

1. The reaction between ferricyanide and egg albumin in solutions of urea, guanidine hydrochloride, and Duponol has been investigated.

2. In neutral medium ferricyanide oxidizes all the SH groups of egg albumin that give a color reaction with nitroprusside. In neutral medium ferricyanide appears to react only with the SH groups of egg albumin. The quantity of ferrocyanide formed can accordingly be considered the equivalent of the number of SH groups in egg albumin detectable with nitroprusside.

3. In solutions of urea, guanidine hydrochloride, and Duponol sufficiently concentrated so that all the egg albumin present is denatured, the same number of SH groups are found—equivalent to a cysteine content of 0.96 per cent.

4. In denaturation of egg albumin loss of solubility (solubility not in presence of the denaturing agent, but solubility examined in water at the isoelectric point) and appearance of reactive SH groups are integral parts of the same process. As denaturation proceeds in urea, SH groups are liberated only in the egg albumin with altered solubility and in this albumin the maximum number of SH groups is liberated. In a molecule of egg albumin either all of its SH groups that give a test with nitroprusside are liberated or none of them are.

EXPERIMENTAL

The egg albumin used in most of these experiments was prepared by La Rosa's method and then recrystallized three times (15). The albumin used in one experiment was prepared by the method of Kekwick and Cannan (13). Before being used a sample of egg albumin was dialyzed in a rocking dialyzer until completely free of ammonium sulfate. Concentration of egg albumin was then determined by drying to constant weight at 105°. The albumin solution was stored in the cold without preservative for the few days during which it was used. Solutions of ferricyanide were used within 3 or 4 days after being made up and during this time were kept in the dark at 1°C.

Deproteinization

Before estimating the quantity of ferrocyanide formed in the egg albumin solution it is necessary (except in the presence of Duponol) to remove the protein. This is done with tungstic acid. A 10 per cent stock solution of sodium tungstate is acidified whenever tungstic acid is needed. To 1.0 cc. of the sodium tungstate solution are added 40 cc. water, 0.70 cc. of 1 N H_2SO_4, and enough water to bring the volume to 50 cc. In presence of Duponol, ferric sulfate does not precipitate protein.

Estimation of Ferrocyanide as Prussian Blue

Prussian blue is formed when ferric sulfate is added to an acidified solution of ferrocyanide. There is a tendency for Prussian blue to precipitate. This can be prevented by adding gum ghatti (7). It is convenient to prepare a solution of ferric sulfate in gum ghatti, as described by Folin and Malmros. To 5 cc. of deproteinized (or Duponol containing) solution are added 0.05 cc. of 0.2 M potassium ferricyanide, 1 cc. of ferric sulfate-gum ghatti and then after 5 minutes, 6.5 cc. water. After standing 5 more minutes Prussian blue is estimated in a photoelectric colorimeter of the Evelyn type, using a red filter (Corning No. 241). A red filter is used because the ferricyanide present does not absorb red light to a significant extent, and ferricyanide is present since an excess is added to the albumin. Still more ferricyanide is added at the time of Prussian blue formation because it was found that when minute quantities of ferrocyanide are being estimated, the amount of Prussian blue formed (in the time interval employed) is increased if ferricyanide is present. With the quantity of ferricyanide added the maximum amount of Prussian blue is formed.

To establish a relationship between intensity of color and quantity of ferrocyanide, Prussian blue is formed in solutions containing known quantities of ferrocyanide. In analytical chemistry standard solutions of ferrocyanide are ordinarily considered to be stable. The solutions required in the present experiments are far more dilute (0.0002 M) than those usually used and these dilute solutions of potassium ferrocyanide are not stable. They must be prepared from more concentrated (0.1 M) stock solutions or from solid potassium ferrocyanide whenever an experiment is done. Prussian blue is formed from known quantities of ferrocyanide under precisely the same conditions as when unknown quantities are present. The 5 cc. of solution, to which 1 cc. of ferric sulfate-gum ghatti subsequently is added, contains between 0.50 and 2.5 cc. of 0.0002 M ferrocyanide. Also included in the 5 cc. of solution are 0.05 cc. 1 N H_2SO_4, 0.05 cc. 0.2 M K ferricyanide, and 2.5 cc. tungstic acid. Of these reagents only ferricyanide influences the color intensity of the blue solution finally obtained. Urea, Duponol, and guanidine hydrochloride have also been added to solutions containing known quantities of ferrocyanide. The quantity of Duponol present in the experiments with egg albumin does not affect the intensity of color when Prussian blue is formed. Urea and guanidine hydrochloride do affect the intensity of color, and it is accordingly necessary to have urea and guanidine hydrochloride in the standard ferrocyanide solutions when these reagents are added to egg albumin.

Reactions between Egg Albumin and Ferricyanide

1. In Urea.—To 0.25 cc. of a 7 per cent albumin solution are added 0.05 cc. 1 M KH_2PO_4-K_2HPO_4 pH 6.7 buffer, 0.05 cc. 0.1 M K ferricyanide, and 350 mg. of urea (100 mg. urea added to each 0.10 cc. of albumin containing solution). A number of these solutions are prepared and kept for various periods of time, some at 25°C., others at 37.5°. After the reaction with ferricyanide, to each solution are added 10 cc. tungstic acid, 0.4 cc. 1 N H_2SO_4, and enough water to bring the volume to 20 cc. The mixture is filtered and 5 cc. of the clear filtrate are taken for Prussian blue formation. Instead of removing egg albumin by precipitation with tungstic acid, the albumin can be left in

solution if Duponol is added for this prevents protein precipitation when ferric sulfate is added. After the reaction between albumin and ferricyanide, 15 cc. of water and 0.4 cc. 1 N H_2SO_4 are added. As the acid is mixed with the protein solution a fine precipitate appears. This clears up at once when 0.5 cc. of a 10 per cent Duponol solution is added. The solution is brought to a volume of 20 cc. by addition of water. When Duponol is added to a strongly acid mixture of egg albumin and ferricyanide, no reaction between protein SH groups and ferricyanide occurs.

Effect of Iodoacetamide and Mercuric Chloride.—To 1 cc. of albumin are added 0.3 cc. phosphate buffer, 0.65 cc. H_2O, 25 mg. iodoacetamide, and 2.0 gm. urea. After standing for an hour at 25°C., 0.5 cc. ferricyanide is added and 30 minutes later the albumin is precipitated with tungstic acid. No Prussian blue forms in the filtrate when ferric sulfate is added. Iodoacetamide does not interfere with Prussian blue formation when it is present in a ferrocyanide solution to which ferric sulfate is added. In another experiment 0.2 cc. of 0.1 M $HgCl_2$ is added instead of iodoacetamide. No Prussian blue is found in this case either.

Albumin in Urea Oxidized by Ferricyanide and Subsequently Treated with Guanidine Hydrochloride or Duponol

To 1.25 cc. albumin are added 0.5 cc. phosphate buffer, 0.25 cc. ferricyanide, and 2 gm. urea. After 30 minutes the albumin is precipitated with tungstic acid and the suspension is centrifuged. The protein precipitate is washed with tungstic acid until it is colorless. To the precipitate are added 0.4 cc. 1 M K_2HPO_4, 1 cc. of a 10 per cent Duponol solution, 0.1 cc. ferricyanide, and water to bring the volume to 15 cc. At 37.5° this mixture forms a clear solution. After 20 minutes the solution is acidified with 1 N H_2SO_4 and diluted with water to 20 cc. Of this solution 5 cc. are taken to test for Prussian blue formation, but no color appears. In another experiment guanidine hydrochloride instead of Duponol is added to the tungstic acid precipitate of albumin. The precipitate is packed down hard in the centrifuge and to it are added 0.3 cc. 1 M K_2HPO_4 and 300 mg. guanidine hydrochloride. A small part of the mixture is tested with nitroprusside. No color is observed. To the rest of the albumin-guanidine hydrochloride mixture is added 0.05 cc. ferricyanide. After 30 minutes at 25°, 10 cc. of tungstic acid, 0.5 cc. H_2SO_4, and water are added to bring the volume to 20 cc. No Prussian blue appears in the filtrate when ferric sulfate is added.

Test for Completeness of Denaturation.—To 1 cc. of the albumin solution 1 gm. of urea is added. After the solution has stood at 25° for 30 minutes it is diluted to 10 cc. To 5 cc. are added 0.25 cc. of a 2 M pH 4.7 acetate buffer and 1.25 cc. saturated $(NH_4)_2SO_4$. The suspension is filtered. Only a slight haze appears in the filtrate when trichloracetic acid is added.

Oxidation of Albumin in Insufficient Urea to Produce Complete Denaturation

To 15.5 cc. of albumin solution are added 1.5 cc. of phosphate buffer, 3.0 cc. of 0.1 M ferricyanide, and 13 gm. of urea. The solution remains at 25° for 20 minutes. A heavy precipitate is formed when 20 cc. of a pH 4.7, 1 M acetate buffer are added and with this

suspension 10 cc. of a saturated ammonium sulfate solution are mixed. A clear supernatant is obtained after centrifuging. The supernatant solution is dialyzed at 1° in a rocking dialyzer against distilled water for 24 hours. It is then completely free of ferricyanide, but somewhat turbid. This fluid is filtered and the protein content of the clear filtrate is determined by drying in an oven at 105°. Each cc. contains 8.52 mg. of protein. Since there are 64 cc. of this solution (561.3 mg. in all) and since the 15.5 cc. of albumin solution used at the beginning of the experiment contained 70.2 mg. per cc. (1088 mg. in all), somewhat less than 50 per cent of the albumin originally present was denatured by urea. SH groups in the dialyzed egg albumin solution are estimated by adding to 2 cc. of the solution 0.05 cc. phosphate buffer, 0.05 cc. of 0.1 M ferricyanide, and 0.5 cc. of a 10 per cent Duponol solution and then proceeding as described below. The albumin precipitated from urea solution by adding acetate buffer is washed with a $\frac{1}{2}$ saturated $(NH_4)_2SO_4$ solution, removing the washings by centrifuging, until the protein precipitate is free of the yellow color of ferricyanide and the washings contain no protein precipitable with trichloracetic acid. The protein precipitate is then tested for SH groups with nitroprusside and ammonium hydroxide in the presence of guanidine hydrochloride. The test is negative.

2. In Guanidine Hydrochloride.—300 mg. guanidine hydrochloride are dissolved in 0.25 cc. albumin. The solution remains at 25° for 30 minutes and then to it are added 0.05 cc. of 1 M pH 6.8 potassium phosphate buffer and 0.05 cc. of 0.1 M ferricyanide. After the reaction with ferricyanide the protein is precipitated by adding 10 cc. tungstic acid and 0.4 cc. of 1 N H_2SO_4. The suspension is diluted with water to a volume of 20 cc., shaken, and filtered. Of the filtrate 5 cc. are taken for Prussian blue formation. The conditions of the reaction between ferricyanide and egg albumin in guanidine hydrochloride are varied: the temperature is either 25 or 37.5°; tungstic acid is added to the solution at intervals varying from 5 to 80 minutes after mixing ferricyanide with the albumin; the concentration of ferricyanide added is either 0.1 M or 0.5 M; the quantity of guanidine hydrochloride added is either 200 or 300 mg.; ferricyanide, phosphate buffer, and albumin are mixed together before adding guanidine hydrochloride. None of these variations affects the quantity of ferricyanide reduced. The phosphate buffer used can vary in pH from 6.1 to 7.3, or even 1 M K_2HPO_4 can be used, without affecting the reaction, but if an acetate buffer of pH 4.7 is used, less ferricyanide is reduced.

Effects of Iodoacetamide and Mercuric Chloride.—After dissolving 300 mg. of guanidine hydrochloride in 0.25 cc. of albumin, 0.05 cc. of phosphate buffer is added. In one experiment 20 mg. of iodoacetamide are added to this solution and in another experiment 0.05 cc. of 0.1 M $HgCl_2$ is added. After standing for an hour at 25°C., 0.05 cc. of ferricyanide is added to each albumin-guanidine hydrochloride mixture and after another hour tungstic acid is added. No blue color appears in the filtrate when ferric sulfate is added.

Recovery of Ferrocyanide Added to Egg Albumin in Guanidine Hydrochloride.—The egg albumin in 0.25 cc. is oxidized by ferricyanide in the presence of guanidine hydrochloride and then precipitated by tungstic acid. The precipitated albumin is separated by centrifuging, washed free of ferricyanide with tungstic acid, and then washed with 0.1 M pH 6.8 phosphate. To the precipitated albumin are added 300 mg. guanidine hydrochloride, 0.05 cc. of 1 M phosphate, 0.05 cc. of ferricyanide, and finally 1 cc. of 0.001 M ferrocyanide. After standing for 10 minutes, tungstic acid and water to bring the volume to 20 cc. are

added and the suspension is filtered. The quantity of Prussian blue formed by the ferrocyanide in 5 cc. of the filtrate is compared with the quantity formed in 5 cc. of a control solution made by adding 1 cc. of ferrocyanide to 10 cc. of tungstic acid, 0.05 cc. of ferricyanide, and 8.5 cc. of water. The galvanometer readings of the colorimeter were practically identical for the two Prussian blue solutions showing that all of the ferrocyanide added to the egg albumin was recovered.

Completeness of Denaturation in guanidine hydrochloride was demonstrated as it was in urea: 300 mg. of guanidine hydrochloride were dissolved in 0.25 cc. of albumin. After standing for 15 minutes, 4 cc. of water, 0.25 cc. of a 2 M pH 4.7-acetate buffer, and 1 cc. of saturated $(NH_4)_2SO_4$ are added. The precipitate is filtered off. Trichloracetic acid is added to the precipitate. No sign of a protein precipitate is detectable.

3. In Duponol.—To 0.25 cc. of the albumin solution are added 0.05 cc. of pH 6.8 1 M phosphate buffer, 2 cc. of water, 0.5 cc. of a 10 per cent Duponol solution, and 0.05 cc. of ferricyanide. The solution is kept at 37.5° for 10 minutes. It is then acidified by the addition of 0.4 cc. 1 N H_2SO_4 and diluted to 20 cc. with water. 5 cc. of this solution are taken for Prussian blue formation.

Effects of Iodoacetamide and Mercuric Chloride.—To 1.5 cc. of the albumin solution are added 0.2 cc. of phosphate buffer, 0.5 cc. of Duponol, and either 25 mg. of iodoacetamide or 0.2 cc. of 0.1 M $HgCl_2$. After 10 minutes at 37° 0.05 cc. of ferricyanide is added. The solution remains at 37.5° for 30 minutes before acidifying and diluting to 20 cc. No Prussian blue appears in the filtrate when ferric sulfate is added.

Completeness of Denaturation.—To 2 cc. of albumin solution are added 10 cc. of water and 4 cc. of Duponol. The solution is placed in a cellophane tube and dialyzed against distilled water in a rocking dialyzer for 36 hours at 37.5°. At the end of this time the albumin solution remains clear. To 3 cc. of the solution is added 0.05 cc. of a saturated ammonium sulfate solution. A heavy precipitate forms. After filtration 0.5 cc. of 50 per cent trichloracetic acid is added to the clear filtrate. No turbidity appears.

BIBLIOGRAPHY

1. Anson, M. L., *J. Gen. Physiol.*, 1939, **23,** 247.
2. Anson, M. L., *J. Biol. Chem.*, 1940, **135,** 797.
3. Anson, M. L., and Mirsky, A. E., *J. Gen. Physiol.*, 1929, **13,** 121.
4. Arnold, V., *Z. physiol. Chem.*, 1911, **70,** 300, 314.
5. Balls, A. K., and Lineweaver, H., *J. Biol. Chem.*, 1939, **130,** 669.
6. Brand, E., and Kassell, B., *J. Biol. Chem.*, 1940, **133,** 437.
7. Folin, O., and Malmros, H., *J. Biol. Chem.*, 1929, **83,** 115.
8. Greenstein, J. P., *J. Biol. Chem.*, 1938, **125,** 501.
9. Greenstein, J. P., *J. Biol. Chem.*, 1939, **128,** 233.
10. Greenstein, J. P., *J. Biol. Chem.*, 1939, **130,** 519.
11. Greenstein, J. P., *J. Nat. Cancer Inst.*, 1940, **1,** 77.
12. Kassell, B., and Brand, E., *J. Biol. Chem.*, 1938, **125,** 435.
13. Kekwick, R. A., and Cannan, R. K., *Biochem. J.*, London, 1936, **30,** 227.
14. Kuhn, R., and Desnuelle, P., *Z. physiol. Chem.*, 1938, **251,** 14.
15. La Rosa, W., *Chem. Analyst*, 1927, **16,** 3.
16. Mason, H., *J. Biol. Chem.*, 1930, **86,** 623.

17. Mirsky, A. E., and Anson, M. L., *J. Gen. Physiol.*, 1935, **18**, 307.
18. Mirsky, A. E., and Anson, M. L., *J. Gen. Physiol.*, 1936, **19**, 427.
19. Mirsky, A. E., and Anson, M. L., *J. Gen. Physiol.*, 1936, **19**, 439, 559.
20. Mirsky, A. E., and Anson, M. L., *J. Gen. Physiol.*, 1936, **19**, 451.
21. Ramsden, W., *Tr. Faraday Soc.*, 1913, **9**, 95.
22. Rosner, L., *J. Biol. Chem.*, 1940, **132**, 657.
23. Stern, K. G., and White, A., *J. Biol. Chem.*, 1937, **117**, 95.
24. Todrick, A., and Walker, E., *Biochem. J.*, London, 1937, **31**, 292.
25. Young, E. G., *Proc. Roy. Soc. London, Series B*, 1922, **93**, 15.

SULFHYDRYL GROUPS IN FILMS OF EGG ALBUMIN

By A. E. Mirsky

(*From the Hospital of The Rockefeller Institute for Medical Research*)

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The shape of the protein molecule changes completely when a protein spreads to form a unimolecular film at an interface. The egg albumin molecule, for example, may be considered to be an ellipsoid in solution with a major axis of 91 A.u. and a minor axis of 32 A.u. (10). In the film it flattens out to a thickness of only 8 A.u. (4). There are groups in native soluble egg albumin which do not react with certain reagents. The SH groups of denatured egg albumin give a color reaction with nitroprusside, reduce ferricyanide, and react with iodoacetate in much the same manner as do the SH groups of cysteine (8). In native egg albumin the SH groups do not show these reactions. The present investigation deals with the activity of the SH groups in thin films of egg albumin. Information concerning the SH groups in the protein film throws light on two problems:

1. In a large molecule such as that of egg albumin, 32 A.u. in diameter, many atoms will occupy inner positions in the elaborate structure. The arrangement of the peripheral groups of atoms may be so compact as to constitute a barrier preventing contact between inner groups of atoms and many of the substances dissolved in the medium in which the protein itself is dissolved. There would, according to this view, be an *interior* of the protein molecule which is definitely less accessible to many reagents than the periphery of the molecule. The structure of the protein molecule may, on the other hand, be so loosely knit that there is no significant distinction between peripheral and internal groups, at least in so far as concerns accessibility to reagents in the surrounding media. When egg albumin spreads at an interface the film of protein is so thin that there can hardly be said to be an "interior" of the molecule. In the film all groups are on the periphery. The protein molecule in forming a film may be said to unfold. If groups inactive in the native egg albumin molecule become active when a film is formed, this is an indication that the groups have become exposed due to the unfolding process—an indication that the peripheral groups of the native, unchanged, egg albumin molecule act as a barrier between reagents in the surrounding solution and groups in the interior

of the protein molecule. Evidence for the existence of such an interior is provided by investigation of SH groups in films of egg albumin.

2. The protein in the film is insoluble. If the film of protein is rolled up and immersed in the medium in which it had been dissolved, it no longer dissolves. In this respect it resembles protein that has been denatured by heat, alcohol, acid, alkali, urea, guanidine hydrochloride, and other agents, and it has indeed been suggested that the protein in the film is denatured (2, 9). One of the characteristics of denaturation is the appearance of active groups. In the denaturation of egg albumin the appearance of active SH groups and the formation of insoluble protein (insoluble in water at the isoelectric point) are integral parts of the same process. The appearance of these groups in the film of egg albumin would indicate that this protein too is denatured; that when a film is formed the same fundamental change in the protein occurs as when it is heated or dissolved in urea, guanidine hydrochloride, or Duponol. Estimation of SH groups in films of egg albumin does in fact provide an insight into the whole process of protein denaturation.

Method

SH groups in the films are estimated by their reaction with ferricyanide. It has been shown that in neutral medium the quantity of ferricyanide reduced by egg albumin may be taken as the equivalent of the number of active SH groups present (7). The quantity of ferrocyanide formed by the SH groups in a single protein film (of practicable area) would be less than can be estimated by methods now available. For each experiment a great many films must be used. These are easily prepared. At the surface of an egg albumin solution there is always a film of protein and when the film is removed another forms. By continually shaking an albumin solution, the films that form are constantly removed until eventually all the protein originally in solution is in the form of clumped together, insoluble films. In this way large quantities of "film protein" can be had. The reaction between ferricyanide and film protein can take place under two different conditions. In one procedure the ferricyanide is mixed with a certain quantity of clumped together film protein, previously prepared by shaking an albumin solution. The finely divided suspension is constantly agitated to promote mixing. In another procedure ferricyanide is added to the egg albumin solution before the film protein is prepared. Then while the solution is shaken the ferricyanide is on the spot to react with each film while it is still a surface film. As the ferricyanide-albumin solution is shaken, more and more films are formed. At any given time the reaction

may be stopped by adding tungstic acid. The quantity of ferrocyanide in the protein-free filtrate is then estimated. In another sample of albumin the quantity of insoluble protein formed at that time is estimated. In procedure I, ferricyanide reacts with albumin only after it has been removed from the surface; in procedure II, ferricyanide is able to react with albumin while it still is at the surface.

RESULTS AND DISCUSSION

It can be seen from Table I that when ferricyanide is present to react with the films of egg albumin while they are being formed (procedure II) the results are precise and definite. The quantity of ferrocyanide formed for each milligram of insoluble egg albumin is constant, although the time of reaction and the amounts of insoluble albumin vary considerably. The quantity of ferrocyanide formed per milligram of insoluble albumin is the same at pH 6.9 as at pH 7.4, but more than at pH 6.6. Less ferricyanide is reduced at pH 6.6 because some of the SH groups fail to take part in the reaction, as tests with nitroprusside demonstrate. The tests are made on insoluble egg albumin that has been washed free of soluble egg albumin and ferricyanide. When samples of precipitated egg albumin from experiments at pH 6.6, 6.9, and 7.4 are tested with nitroprusside and ammonium hydroxide, in no case is a color obtained. When the same tests are carried out in the presence of guanidine hydrochloride an intense color appears in the albumin from the experiment at pH 6.6 and only just detectable colors in the albumin from the experiments at pH 6.9 and 7.4. It is characteristic of SH groups in general that the more acid the solution in which they are placed the less readily do they react. It is also characteristic of an SH group that its hydrogen is readily displaced by mercury. If mercuric chloride is added to a cysteine solution or to denatured egg albumin in solutions of urea, guanidine hydrochloride, or Duponol, these solutions no longer reduce ferricyanide (7). Nor do films of egg albumin reduce ferricyanide if mercuric chloride is present.

The number of SH groups found in films of egg albumin (by procedure II) is the same as in egg albumin denatured by solutions of urea, guanidine hydrochloride, or Duponol (7). Only in heat denatured egg albumin does the number of active SH groups seem to be different—0.5 to 0.6 per cent (8, 11, 6, 5, 1). This apparent difference is due to a curious oversight: In egg albumin denatured by urea, guanidine hydrochloride, Duponol, or rendered insoluble by surface forces, SH groups are estimated while the denaturing agent is still present (that is to say, estimations are made on albumin in a solution of the denaturing reagent or on albumin actually at

the surface), whereas in heat denatured egg albumin SH groups are estimated after the denaturing agent is removed (that is to say, on albumin

TABLE I

Reaction of Ferricyanide with Films of Egg Albumin

Experiment No.	pH	Time of shaking	Albumin in solution	Precipitated albumin	Ferrocyanide formed	SH groups	SH groups in insoluble albumin
		min.	*mg.*	*mg.*	*mM*	*mg. cysteine*	*per cent cysteine*
I	6.91	0	18.5	0	0	0	—
		360	13.5		0.000420		
			13.6	5.0	0.000428	0.051	1.02
			13.5				
			Av. 13.53		Av. 0.000424		
		430	12.55		0.000497		
			12.30	6.0	0.000460	0.058	0.97
			12.75		0.000497		
			Av. 12.53		Av. 0.000485		
		510	11.6		0.000557		
			11.6	7.1	0.000557	0.066	0.93
			11.0		0.000537		
			Av. 11.4		Av. 0.00055		
II	6.91	0	18.5	0	0	0	—
		370	12.6	5.9	0.000464	0.056	0.95
		440	11.5	7.0	0.000540	0.065	0.93
		510	11.25	7.25	0.000568	0.068	0.94
III		0	18.5	0	0	0	—
	6.56	300	11.3	7.2	0.00047	0.056	0.78
		375	10.05	8.45	0.00061	0.073	0.86
		435	9.25	9.25	0.00063	0.076	0.82
IV	7.44	0	18.5	0	0	0	—
		380	14.25	4.25	0.000332	0.040	0.94
		510	13.55	4.95	0.000405	0.0485	0.98
		630	12.55	5.95	0.000458	0.055	0.93

that has been allowed to cool off). If SH groups are estimated in heat denatured egg albumin while the albumin is being heated, precisely the same number of SH groups is found as when estimations are made in the presence of urea, guanidine hydrochloride, Duponol, or surface forces.

In denaturation by heat, if the process is stopped while some egg albumin

still remains in solution, it is found that SH groups appear only in the albumin rendered insoluble, there being none in the albumin that was heated but not yet rendered insoluble. In denaturation by urea SH groups appear only in the fraction of protein with altered solubility (7). When films of insoluble egg albumin are constantly being formed, the fraction of albumin that still is soluble does not reduce ferricyanide. In thermal and urea

TABLE II

Reaction of Ferricyanide with Films of Egg Albumin after They Have Been Removed from the Surface. (Before Adding Ferricyanide the Albumin Had Been Shaken for 25 Hours and 63 Per Cent of It Had Been Coagulated)

Temperature 0–1°C.; pH 6.9

Time of reaction with ferricyanide	Soluble albumin	Insoluble albumin	Ferrocyanide formed	SH groups	SH groups in insoluble albumin
hrs.	*mg.*	*mg.*	*mM*	*mg. cysteine*	*per cent cysteine*
6½	6.85	11.65	0.000342	0.41	0.35
20	5.5	13.0	0.000572	0.687	0.53
42*	4.45	14.05	0.000745	0.895	0.64

* Total SH in albumin at this time, obtained by heating to 85° for 10 minutes. 0.00142 mM ferrocyanide was obtained. This is equivalent to 1.7 mg. cysteine or 0.92 per cent.

TABLE III

Reaction of Ferricyanide with Soluble Heat Denatured Egg Albumin

Temperature 0–1°C.; pH 6.9

Time of reaction with ferricyanide	Ferrocyanide formed	SH groups	SH groups in albumin
hrs.	*mM*	*mg. cysteine*	*per cent cysteine*
1	0.00064	0.77	0.415
5	0.000828	0.993	0.54
6	0.000865	1.04	0.56
8	0.00087	1.045	0.565

denaturation and in the process of film formation, appearance of SH groups and alteration in solubility are integral parts of the same process.

When ferricyanide reacts with films of egg albumin after they have been removed from the surface (procedure I, Table II) the number of SH groups (0.6 per cent) found is the same as in egg albumin denatured by heat and then allowed to cool before SH groups are estimated (Table III). In both cases there is no sharp end-point to the reaction; it appears to continue almost indefinitely, although after some time at a much diminished rate. If at this time ferricyanide is washed away and the protein is tested with nitroprusside, no color reaction is obtained. Still the protein continues

to reduce ferricyanide, although very slowly indeed—as if SH groups were still present in the protein, but not fully accessible to ferricyanide. And there still are SH groups in the protein, for if the nitroprusside test is carried out in the presence of guanidine hydrochloride an intense color is obtained. The SH groups have not been lost by oxidation. The unreactive (or very sluggishly reactive) SH groups of films of egg albumin removed from the surface and the SH groups of heat denatured egg albumin, allowed to cool off, reduce ferricyanide rapidly if the ferricyanide-protein mixtures are heated to 85°. When egg albumin is denatured by a concentrated urea solution and the urea is subsequently diluted, the SH groups of the dissolved albumin no longer reduce ferricyanide rapidly (as they do in concentrated urea, the reaction being complete in less than 1 minute), but in the same sluggish and incomplete manner as in albumin denatured by heat and then cooled.[1] When the albumin in such a dilute urea solution is heated or when urea is added to the solution the SH groups immediately reduce ferricyanide.

The properties of egg albumin denatured by heat and urea that have just been briefly noted will be more completely described in another paper. They have been referred to in this paper because they show a close resemblance between surface films of egg albumin that have been removed from the surface, egg albumin denatured by heat and then allowed to cool, and egg albumin denatured by concentrated urea solution and the urea then diluted. There is also a close resemblance between the properties of films of egg albumin while they still are at the surface, albumin denatured by heat and kept heated, and albumin in solutions of urea, guanidine hydrochloride, or Duponol sufficiently concentrated to cause denaturation. In the many different ways of denaturing egg albumin the two most characteristic changes in the protein are the loss of solubility at the isoelectric point and the appearance of SH groups. These two changes also take place when a film of egg albumin is formed. In a film precisely the same number of SH groups appear as when egg albumin is denatured; and when the films are withdrawn from the surface, and allowed to clump together, the same change in SH groups takes place as when the denaturing agent is withdrawn from denatured egg albumin.

It is clear that the film at the surface of an egg albumin solution should

[1] When SH groups of denatured egg albumin disappear no native egg albumin is formed. All of the protein is still insoluble in water at the isoelectric point. That the amount of ferricyanide reduced by heat denatured egg albumin is increased by increasing the ferricyanide concentration or the time of reaction has been noted by Anson (1). The observations reported in the present paper were made before the publication of his paper.

be considered to be denatured. Apparently the same fundamental change takes place when a film of egg albumin is formed as when albumin is modified by heat, urea, and other agents. This would explain why the properties of a film of protein are not changed by heating to a temperature well above the temperature of denaturation of the same protein in solution (3); the change wrought in dissolved albumin by heat had already taken place when the film was formed.[2] The change in configuration of the egg albumin molecule that occurs when a film forms can in a general way be clearly described; the film, as has been said, is only 8 A.u. thick whereas the molecule in solution is an ellipsoid with a major axis of 91 A.u. and a minor axis of 32 A.u. This change in configuration would explain why SH groups appear in egg albumin. Groups in the interior of the protein molecule become exposed in a film and are thus able to take part in the reaction from which they had previously been shielded by the peripheral groups of the native protein molecule. The egg albumin molecule unfolds when a film is formed.[3] Unfolding, and uncovering of the interior of a molecule, may also be supposed to occur whenever the SH groups of egg albumin give a reaction with nitroprusside and reduce ferricyanide—as they do when egg albumin is denatured by every agent that has so far been investigated. In another paper direct evidence will be given for the unfolding of egg albumin in concentrated solutions of urea.

Many SH groups in the films of egg albumin no longer reduce ferricyanide when the films are clumped together. These groups have not been oxidized. They simply are inaccessible to the ferricyanide. The albumin at this time is in the form of a suspension and (although the suspension is finely

[2] Dognon and Piffault who made the observation referred to (that a film of serum albumin heated to 70° is not changed) concluded that a thin film of albumin is not denatured by heat.

[3] When the egg albumin molecule unfolds SH groups could conceivably react with ferricyanide for a reason quite different from the one that has just been given. If two SH groups must react simultaneously with ferricyanide to give one S—S group, it must be supposed that the two SH groups are placed close together in the protein molecule. It may be that in the native egg albumin molecule SH groups are so far separated from each other that it is impossible for two of them to react with ferricyanide to yield a S—S group. Unfolding would make possible the reaction with ferricyanide by bringing SH groups close together. This explanation is untenable because the SH groups of native egg albumin do not react with iodoacetate, and in this reaction SH groups do not react in pairs.

It has also been supposed that SH groups do not exist as such in the native protein molecule, that during denaturation they are formed from S—S groups. This theory will be considered in another paper.

divided) this may seem to be a sufficient reason for the inaccessibility of some SH groups to ferricyanide. Even in a *solution* of egg albumin, however, SH groups may become inaccessible to ferricyanide. This happens in a concentrated urea solution when the urea is diluted. And when heat denatured albumin is cooled its SH groups become inaccessible whether the albumin is dissolved or precipitated. An explanation for the disappearance of SH groups will be offered in another paper, when the phenomenon itself will be more completely described.

SUMMARY

1. The same number of SH groups reduces ferricyanide in surface films of egg albumin as in albumin denatured by urea, guanidine hydrochloride, Duponol, or heat, provided the ferricyanide reacts with films while they still are at the surface and with the denatured proteins while the denaturing agent (urea, heat, etc.) is present.

2. The SH groups of a suspension of egg albumin made by clumping together many surface films react with ferricyanide in the same sluggish and incomplete manner as do the groups in egg albumin denatured by concentrated urea when the urea is diluted or in albumin denatured by heat when the solution is allowed to cool off.

3. The known change in configuration of the egg albumin molecule when it forms part of a surface film explains why SH groups in the film react with ferricyanide whereas those in native egg albumin do not. In the native egg albumin molecule groups in the interior are inaccessible to certain reagents. A film is so thin that there are no inaccessible groups.

4. Because of the marked resemblance in the properties of egg albumin in surface films and of egg albumin after denaturation by the recognized denaturing agents, it may be supposed that the same fundamental change takes place in denaturation as in film formation—indeed, that film formation is one of the numerous examples of denaturation. This would mean that in general the SH groups of denatured egg albumin reduce ferricyanide and react with certain other reagents because they are no longer inaccessible to these reagents.

EXPERIMENTAL

The reagents used have been described in a previous paper (7). All experiments, unless otherwise noted, were done at 0 to 1°.

Procedure I.—In each of twenty-four 60 cc. glass stoppered pyrex bottles are placed 0.20 cc. of egg albumin solution (containing 18.48 mg. of egg albumin), 0.10 cc. of a 1 M pH 6.9 KH_2PO_4-K_2HPO_4 buffer, and 9.2 cc. of water. The bottles are placed in a shaker making 240 strokes per minute. The shaker is placed in a cold room kept at a

fraction of a degree above zero. Even after shaking for more than 24 hours only two-thirds of the egg albumin is coagulated. Shaking is done in the cold to reduce to a minimum oxidation by the air in the bottles. (Actually little or no oxidation takes place. After shaking in the absence of ferricyanide for 25 hours and then with ferricyanide added for 45 hours the mixture was heated to 85° for 10 minutes. The quantity of ferrocyanide formed was the same as in a mixture heated at once, without any previous shaking.) After shaking for 25 hours, 0.10 cc. of 0.2 M ferricyanide is added to half of the bottles and all the bottles are replaced in the shaking machine. At various intervals of time four bottles (two with and two without ferricyanide) are removed from the shaker. In the mixtures containing ferricyanide the protein is precipitated by adding 0.3 cc. of 1 N H_2SO_4 and 0.1 cc. of a 10 per cent sodium tungstate solution. The protein is filtered off and the quantity of ferrocyanide is estimated, as previously described. To the albumin mixtures not containing ferricyanide 0.4 cc. of water is added and the suspension is then filtered. The protein content of the filtrate is estimated by the Kjeldahl method. From this the quantity of protein coagulated is reckoned by difference.

Comparison with Reduction of Ferricyanide by Heat Denatured Egg Albumin.—To 2 cc. of egg albumin solution are added 15.8 cc. of water and 2.2 cc. of 0.06 N HCl. The solution is heated at 85° for 10 minutes and is then cooled in an ice mixture. To 2 cc. of this solution (containing 18.48 mg. egg albumin) are added 7.4 cc. of water, 0.1 cc. of phosphate buffer, and 0.1 cc. of 0.2 M ferricyanide. These solutions of albumin and ferricyanide are shaken (although they are perfectly clear solutions) in the cold room. After various intervals of time to each sample are added 0.3 cc. of H_2SO_4 and 0.1 cc. of sodium tungstate. Ferrocyanide is then estimated in the protein-free filtrate.

Procedure II.—Ferricyanide is present in one-half of the bottles from the beginning of the shaking experiment. Various 1 M K_2HPO_4-KH_2PO_4 buffers are used—at pHs 6.56, 6.91, 7.44. In one experiment, with pH 6.91 buffer, 0.1 cc. of 0.1 M mercuric chloride is added to each sample of egg albumin at the same time that the ferricyanide is added. In this experiment the albumin is shaken for 10 hours.

REFERENCES

1. Anson, M. L., *J. Gen. Physiol.*, 1939, **23**, 247.
2. Bull, H. B., *J. Biol. Chem.*, 1938, **125**, 585.
3. Dognon, A., and Piffault, C., *Compt. rend. Acad. sc.*, 1939, **208**, 654.
4. Gorter, E., and Grendel, F., *K. Akad. Wetensch. Amsterdam, Proc. Sect. Sc.*, 1929, **32**, 770.
5. Greenstein, J. P., *J. Biol. Chem.*, 1938, **125**, 501.
6. Kuhn, R., and Desnuelle, P., *Z. physiol. Chem.*, 1938, **251**, 14.
7. Mirsky, A. E., *J. Gen. Physiol.*, 1941, **24**, 709.
8. Mirsky, A. E., and Anson, M. L., *J. Gen. Physiol.*, 1935, **18**, 307.
9. Mirsky, A. E., and Pauling, L., *Proc. Nat. Acad. Sc.*, 1936, **7**, 439.
10. Neurath, H., *J. Am. Chem. Soc.*, 1939, **61**, 1841.
11. Todrick, A., and Walker, E., *Biochem. J.*, London, 1937, **31**, 292.

THE COURSE OF ROD DARK ADAPTATION AS INFLUENCED BY THE INTENSITY AND DURATION OF PRE-ADAPTATION TO LIGHT*

By CHARLES HAIG

(*From the Research Service, First Medical Division, Welfare Hospital, New York City Department of Hospitals, and the Department of Medicine, College of Physicians and Surgeons, Columbia University*)

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I

The increase in sensitivity of a limited area of the retinal periphery, following pre-exposure to light of varying intensity and duration, has been the subject of a number of recent studies (Müller, 1931; Winsor and Clark, 1936; Wald and Clark, 1937; Hecht, Haig, and Chase, 1937).

While these data have shown that the effect of varying the duration of pre-adaptation is in many ways similar to that of varying the intensity, there has appeared no report of well controlled measurements of the relative influence of these two factors upon the course of the subsequent dark adaptation.

It is our present purpose to describe a series of such measurements obtained in this laboratory.

II

Methods

In the adaptometer employed (Hecht and Shlaer, 1938), which is of the monocular type, the same eye piece is used during both light and dark adaptation periods. This is accomplished by a simple mechanical alteration of the lens and intensity control systems at the termination of the period of light adaptation. As the instrument is not regularly provided with an artificial pupil, for the present study this was supplied by a disc of blackened metal through which was drilled a circular, 2 mm. hole. This was attached to the regular eye piece of the instrument. With the eye in place against the eye piece the observer's cornea was at a distance of 3 mm. from the artificial pupil.

A second method of obtaining a constant pupil was to employ as one of the observers a patient who had an Argyll Robertson pupil due to syphilis. This observer's pupil was

* A preliminary report of these measurements was presented to the American Society of Zoologists in December, 1938 (*Anat. Rec.*, 1938, **72**, suppl., 82), and to the American Physiological Society in April, 1939 (*Am. J. Physiol.*, 1939, **126**, 518).

fixed with respect to light stimuli at a diameter of about 5 mm., and was thus virtually in the open condition. It should be added that, due to cirrhosis of the liver, his visual threshold was about five times higher, and his rate of rod dark adaptation somewhat slower than normal. We found no reason to suspect that either of these pathological conditions caused any fundamental alteration of the essential process of dark adaptation. This conclusion is based, in part, upon the fact that, as one of the subjects of a study of the relation between dark adaptation and vitamin A in cirrhosis of the liver (Haig, Hecht, and Patek, 1938; Patek and Haig, 1939), this patient's dark adaptation was rendered completely normal, in rate as well as in threshold level, by parenteral administration of vitamin A.

The adapting light was white, and its brightness was controlled with neutral decimal filters. In all of the measurements on the normal observer (E. M. H.) the artificial pupil was used during light adaptation as well as during dark adaptation. In those on observer R. C., whose pupils were pathologically fixed, the artificial pupil was not used. Since the threshold, as well as the adapting, intensities recorded are the external ones, it should be kept in mind that they represent comparable retinal illuminations only with reference to the same pupil size.

A violet filter (Corning No. 511) was used in the path of the test light to provide a means of perceptually differentiating between cone (colored) and rod ("colorless") responses. The test light was flashed for 0.2 second at each observation. The retinal region measured was a 3° circular area located 7° nasally to the fovea of the right eye of observer E. M. H. and the left eye of observer R. C.

The general procedure of a dark adaptation run was as follows: The observer became completely dark adapted by a stay of 30 minutes in the dark room in which the test was to be made. He then looked through the exit pupil of the instrument at the fixation light, and the adapting light was turned on for a predetermined period. Immediately upon turning off the adapting light, by shifting the lens and intensity control systems to the threshold measuring position, observations began, and were continued until the threshold reached a relatively constant, low level. Readings were made almost as frequently as possible during the early portions of both cone and rod adaptation, and with decreasing frequency as the processes slowed down. At each observation the observer looked steadily at the fixation light (which was dimmed as adaptation proceeded) and the operator flashed the test light at higher and lower brightness levels until a minimum response was obtained. We have found that by starting the observation with a readily visible stimulus, the operation of placing the eye in exactly proper relation to the fixation and stimulating lights, and to the exit pupil, is greatly facilitated. The final procedure in obtaining a reading was to approach the threshold from below in steps of 0.08 log unit of intensity until a visible flash was observed. Thus, although the observations began in the manner employed for measuring the "disappearance" threshold, the final brightness recorded was a modified "appearance" threshold. The appearance threshold is known to be somewhat higher than the disappearance threshold (Charpentier, 1886).

In addition to reporting the presence or absence of a visible flash, the observer also described the color sensation associated with each threshold reading. We have consistently observed the sharp change in hue accompanying the sudden drop in threshold occurring at the beginning of rod adaptation (Hecht, Haig, and Chase, 1937), but have found, for the two observers of the present experiments, that the change is not from violet to white, but rather from violet to a "gray-blue," which cannot be described as de-

saturated violet. An unequivocally white sensation is not observed until the threshold has descended to about 1.5 logarithmic units of intensity above the final rod threshold. The latter is also true of the exclusively rod observations following low degrees of light adaptation (*cf.* Kroh, 1922).

III

Measurements after Varying Degrees of Light Adaptation

Measurements were made of the dark adaptation of both observers following adaptation to white light of 447 millilamberts intensity for periods

TABLE I

Dark adaptation as measured with 2 mm. artificial pupil following light adaptation for various periods to white light of 447 ml. intensity. Individual observations made at one sitting by observer E.M.H. Time is in minutes; intensity in micromicrolamberts. Values in bold face type appear violet at the threshold.

0.1 min.		0.4 min.		1.0 min.		2.0 min.		4.0 min.		6.0 min.		10.0 min.	
Time	Log *I*	Time	Log *I*	Time	Log *I*	Time	Log *I*	Time	Log *I*	Time	Log *I*	Time	Log *I*
0.4	5.88	**0.3**	**6.85**	**0.5**	**6.63**	**0.2**	**7.41**	**0.3**	**7.46**	**0.7**	**6.89**	**0.2**	**7.41**
0.9	5.28	0.8	5.79	1.0	6.16	**0.8**	**6.58**	**0.8**	**6.75**	**1.0**	**6.77**	**0.5**	**6.94**
1.3	5.06	1.3	5.51	2.1	5.69	**1.3**	**6.47**	**1.3**	**6.52**	**2.1**	**6.30**	**0.8**	**6.82**
1.8	4.77	1.8	5.26	2.7	5.34	2.4	6.11	**2.2**	**6.28**	**3.1**	**6.18**	**1.4**	**6.58**
2.3	4.54	2.9	4.80	3.7	5.13	3.3	5.71	**3.3**	**6.04**	4.5	5.83	**3.7**	**6.16**
4.6	4.42	4.1	4.56	5.0	4.70	4.1	5.48	4.3	5.86	5.7	5.36	5.0	5.64
6.7	4.35	5.7	4.44	6.7	4.46	5.2	5.12	5.6	5.34	7.1	5.08	7.0	5.12
9.3	4.30	8.3	4.35	9.2	4.39	6.5	4.89	6.5	5.17	8.7	4.77	8.5	4.64
		11.1	4.30	12.0	4.30	8.3	4.60	7.8	4.89	10.2	4.67	10.5	4.53
				20.0	4.30	10.7	4.46	9.2	4.58	11.8	4.60	15.2	4.34
						15.3	4.34	10.7	4.51	13.4	4.37	19.1	4.30
						18.7	4.30	12.5	4.46	18.6	4.30		
								14.0	4.35				
								18.0	4.35				
								21.7	4.30				

ranging from 0.1 to 10 minutes (Tables I and II, and Fig. 1). Another set of observations was made of the dark adaptation of both observers following 4 minute exposures to white lights ranging in intensity from 4 to 4700 ml. (Tables III and IV, and Fig. 2).

The entire range of pre-exposure durations or intensities was run through at a single sitting. In the case of observer R. C., this was done twice for each set of measurements and the observations averaged. Unfortunately, the experiments on the influence of duration were made about 4 days after the ones on the influence of intensity. During this period the threshold levels of both observers had changed, that of E. M. H. having risen 0.29

TABLE II

Dark adaptation as measured with pathologically fixed pupil (5 mm.) following light adaptation for various periods to white light of 447 ml. intensity. Average of two observations made in two sittings by observer R.C. Time is in minutes; intensity in micromicrolamberts. Values in bold face type appear violet at the threshold.

0.2 min.		0.5 min.		1.0 min.		1.2 min.		2.0 min.		5.0 min.		6.0 min.		10.0 min.	
Time	Log *I*	Time	Log *I*	Time	Log *I*	Time	Log *I*	Time	Log *I*	Time	Log *I*	Time	Log *I*	Time	Log *I*
0.2	5.67	1.0	5.90	**0.3**	**6.54**	**0.1**	**6.65**	**0.3**	**6.77**	**0.7**	**6.43**	**0.4**	**6.62**	**0.2**	**6.67**
0.8	5.07	1.6	5.43	**0.8**	**6.42**	**0.8**	**6.30**	**0.8**	**6.42**	**1.4**	**6.27**	**1.3**	**6.50**	**0.6**	**6.51**
1.2	4.72	2.0	5.08	2.3	5.71	**2.5**	**6.30**	**2.3**	**6.42**	**3.5**	**6.20**	**2.8**	**6.39**	**1.2**	**6.32**
1.6	4.60	2.4	4.91	3.0	5.19	3.5	5.83	**3.3**	**6.42**	**5.4**	**6.03**	**5.2**	**6.22**	**2.4**	**6.27**
2.4	4.36	3.5	4.60	4.0	4.91	4.8	5.43	**4.7**	**6.18**	6.5	5.84	**6.4**	**6.22**	**5.0**	**6.20**
3.4	4.25	5.1	4.37	5.3	4.67	5.8	5.00	5.6	5.78	7.3	5.61	7.9	5.99	**6.9**	**6.13**
5.1	4.13	7.1	4.20	7.0	4.41	7.7	4.72	6.4	5.55	8.3	5.25	9.3	5.56	9.0	5.96
7.9	4.06	9.7	4.08	9.7	4.25	9.5	4.20	7.7	5.15	9.5	4.97	10.3	5.19	10.1	5.73
11.4	4.01	13.2	4.01	12.6	4.13	11.0	4.08	9.1	4.79	10.7	4.69	11.5	4.81	11.6	5.47
				16.5	4.08	13.7	4.13	10.6	4.56	12.4	4.38	15.2	4.33	12.7	5.08
						15.5	4.01	12.5	4.25	14.6	4.26	18.5	4.20	12.2	4.69
						18.6	4.01	13.9	4.18	16.0	4.15	27.7	4.05	16.1	4.45
								17.1	4.08	19.5	4.10			18.2	4.34
								22.7	4.08					21.1	4.26
														24.7	4.15
														28.3	4.10

TABLE III

Dark adaptation as measured with 2 mm. artificial pupil following light adaptation for 4 minutes to various intensities. Individual observations made at one sitting by observer E.M.H. Time is in minutes; intensity in micromicrolamberts. Values in bold face type appear violet at the threshold.

4 ml.		20 ml.		44 ml.		447 ml.		1150 ml.		2090 ml.		4700 ml.	
Time	Log *I*	Time	Log *I*	Time	Log *I*	Time	Log *I*	Time	Log *I*	Time	Log *I*	Time	Log *I*
0.2	5.12	0.4	5.62	0.5	5.90	**0.3**	**7.00**	**0.3**	**7.84**	**0.2**	**7.84**	**0.5**	**7.84**
0.5	4.89	0.8	5.26	1.0	5.55	**0.9**	**6.41**	**1.3**	**6.89**	**1.0**	**7.48**	**0.8**	**7.48**
0.8	4.53	1.2	4.91	2.2	4.96	1.7	6.06	**2.6**	**6.73**	**1.5**	**7.24**	**2.3**	**7.13**
1.4	4.30	2.4	4.43	3.1	4.60	2.8	5.71	**4.0**	**6.54**	**2.3**	**7.13**	**3.4**	**6.77**
2.2	4.18	3.6	4.20	4.1	4.48	3.7	5.35	5.5	6.18	**3.4**	**6.89**	**5.7**	**6.77**
3.8	4.06	5.2	4.08	5.3	4.37	5.5	5.01	6.4	5.95	**7.3**	**6.42**	**8.1**	**6.77**
8.1	4.01	11.6	4.01	8.7	4.13	7.0	4.60	7.8	5.47	8.7	6.18	**9.7**	**6.61**
				15.6	4.01	8.8	4.32	9.4	5.08	10.0	5.83	10.8	6.30
						11.6	4.20	11.3	4.60	12.0	5.25	12.7	5.59
						14.5	4.08	13.4	4.43	13.5	4.84	14.0	5.36
						20.3	4.01	16.0	4.25	15.0	4.60	15.5	5.08
								22.2	4.13	16.9	4.37	17.6	4.84
										18.2	4.30	20.8	4.48
										21.8	4.20	24.6	4.25
										23.3	4.13	29.3	4.20

THE INFLUENCE OF HOST RESISTANCE ON VIRUS INFECTIVITY AS EXEMPLIFIED WITH BACTERIOPHAGE

By A. D. HERSHEY and J. BRONFENBRENNER

(From the Department of Bacteriology and Immunology, Washington University School of Medicine, St. Louis)

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Parker (1) has shown that the results of infectivity measurements with vaccinia virus may be interpreted as a Poisson distribution of single infective particles among aliquots of the virus obtained by dilution. Thus, if it may be assumed that there exists a quantity of virus invariably necessary and invariably sufficient to produce a lesion in the skin of the rabbit, the behavior on dilution requires this quantity to be a single indivisible particle.

However, if the possibility exists that some independently varying factor influences the appearance of lesions in the inoculated sites, the Poisson distribution is inapplicable, and a different conclusion is reached. In this case the results can only be interpreted as an indication of a particular kind of dose response among the animals tested. Bryan and Beard (2) have called attention to the fact that the single particle response curve has considerable resemblance to the hyperbolic curves characteristic of certain drugs (per cent of positive responses plotted against dosage). Their discussion gives the impression that the reverse is also necessarily true. Actually, two separate requirements must be met if the response to a drug is to resemble the response to single infective particles. First, the distribution of resistance among the animals must be of the extreme skew type, resulting in an approximately symmetrical distribution of logarithms of individual effective doses. In addition, the dispersion of these doses must have a particular value, *e.g.* the ratio between the dose affecting 64 per cent of the population and the dose affecting 16 per cent must be 10.6. Gaddum (3) has collected data for a number of drugs fulfilling the first of these two requirements. In his Table I, twenty-five examples are cited, together with the dispersion measures obtained. The latter are expressed as the standard deviation of the logarithms of individual effective doses, which, for the single particle response curve, is approximately $\frac{1}{2}$ log 10.6 or 0.51. The values tabulated for the twenty-five measurements fall between 0.04 and

0.91, among which only those for acetonitrile, dysentery toxin, and pneumococcus antibody (protective effect), all tested on mice, lie in the neighborhood of 0.51. Apparently it is quite possible for a drug to approximate the single particle response, but it can only do so by a coincidence, dependent on the properties of the drug and also largely influenced by the choice of animal, and the experimental procedure.

We have found a record of three substances tested on the human skin (4), which is of interest in this connection. Of these three, mercuric iodide in

TABLE I

Effect of Volume of Agar on Infectivity of Phage

Aliquot of phage	4 cc. agar			8 cc. agar		
	Actual count	Expected $n = 1$	Expected $n = 2$	Actual count	Expected $n = 1$	Expected $n = 2$
$\times 10^{-7}$ *cc.*						
8.0	±800	847	7600	463	414	3700
2.7	296	282	847	138	138	414
0.9	94	(94)	(94)	46	(46)	(46)
0.3	32	31	10	13	15	5
0.1	7	10	1	6	5	<1

Twenty times the aliquot of phage shown, in 1.0 cc. of broth, was mixed with 4.0 cc., 18–24-hr broth culture of a susceptible coliform species. After allowing the mixture to stand 5 minutes at room temperature, 0.5 cc. amounts were mixed with 4.0 and 8.0 cc., respectively, of 0.7 per cent nutrient agar, and poured into Petri dishes containing 15 cc. of solidified 1.0 per cent agar. Plaques were counted after 18–24 hrs. at 37°C. The counts shown are the mean of three plates.

The expected counts for $n = 1$ are computed on the assumption that plaques result from single particles; *i.e.*, their number is proportional to the concentration of phage. The expected counts for $n = 2$ are computed on the assumption that plaques result from the coordinate action of two particles, so that their number is proportional to the square of the concentration of phage. In either case, the figures in parentheses are the basis for calculation of the remaining values.

The method of plating used here has not previously been described. It was adopted by the authors several years ago to obtain satisfactory counts with small plaque phages and will be discussed in detail in a forthcoming publication.

aqueous solution shows no resemblance to the behavior of the virus, the per cent response curve being sigmoid with respect to dosage. Of the remaining two, applied in vaseline, chrysarobin approximates fairly well, and mercuric chloride extremely well, to the single particle curve. The data for the latter are reproduced on a suitable scale in Fig. 1. It may be seen that there is very little difference between this series of tests with mercuric chloride, where it is inconceivable that the irritation is produced by a single particle, and the results of a typical titration of vaccinia virus.

The correspondence between the result with mercuric chloride and the probable distribution of single particles is not very remarkable, however,

log unit, and that of R. C. having dropped 0.24 log unit. These changes are safely within the known range of day to day variability (0.30 log unit), and do not in any way represent effects attributable to differences in the experimental management of the two sets of measurements, or to the rela-

TABLE IV

Dark adaptation as measured with pathologically fixed pupil (5 mm.) following light adaptation for 4 minutes to various intensities. Average of two observations made in two sittings by observer R.C. Time is in minutes; intensity in micromicrolamberts. Values in bold face type appear violet at the threshold.

4 ml.		20 ml.		44 ml.		110 ml.		200 ml.		447 ml.		1150 ml.		2090 ml.		4700 ml.	
Time	Log I	Time	Log I	Time	Log I	Time	Log I	Time	Log I	Time	Log I	Time	Log I	Time	Log I	Time	Log I
0.3	5.07	0.3	5.55	0.3	5.84	**0.4**	**6.42**	**0.2**	**6.89**	**0.3**	**7.07**	**0.1**	**7.36**	**0.2**	**7.60**	**0.3**	**7.84**
0.7	4.83	0.7	5.20	0.7	5.61	1.2	5.94	**0.6**	**6.65**	**0.9**	**6.77**	**0.4**	**7.13**	**0.7**	**7.30**	**0.5**	**7.54**
1.3	4.64	1.2	4.90	1.2	5.16	2.9	5.51	**1.1**	**6.49**	**2.4**	**6.63**	**0.7**	**6.89**	**1.3**	**7.11**	**1.0**	**7.30**
3.0	4.36	2.5	4.49	3.0	4.64	5.1	4.93	**2.3**	**6.37**	**3.8**	**6.58**	**1.1**	**6.65**	**2.5**	**6.87**	**1.8**	**7.07**
5.1	4.24	4.6	4.37	5.1	4.46	7.4	4.48	3.6	6.06	**5.8**	**6.46**	**2.3**	**6.54**	**4.4**	**6.71**	**3.1**	**6.83**
7.3	4.24	6.7	4.32	7.0	4.31	10.0	4.37	4.6	5.71	7.7	5.99	**4.4**	**6.54**	**5.7**	**6.61**	**4.1**	**6.73**
9.4	4.25	9.5	4.29	9.9	4.28	12.2	4.26	5.7	5.47	8.9	5.57	**6.5**	**6.54**	**9.4**	**6.71**	**5.8**	**6.69**
		14.1	4.25	14.7	4.25	15.0	4.32	7.0	5.15	10.2	5.28	**10.3**	**6.49**	**11.1**	**6.71**	**7.5**	**6.73**
						16.8	4.25	7.8	5.03	12.8	4.78	12.1	6.22	13.9	6.40	**9.6**	**6.69**
								9.5	4.72	17.4	4.43	14.2	5.65	15.2	6.01	**11.8**	**6.61**
								11.0	4.37	23.4	4.32	15.9	5.11	17.0	5.47	13.5	6.52
								13.7	4.32	26.7	4.25	18.0	4.78	18.2	5.25	14.7	6.24
								17.5	4.25			19.8	4.49	20.1	4.78	16.4	5.77
												24.1	4.25	22.3	4.60	17.7	5.35
												28.9	4.23	25.0	4.49	20.3	4.86
												32.5	4.20	28.0	4.40	23.4	4.60
														37.6	4.25	26.5	4.48
																28.8	4.47
																31.6	4.31
																33.8	4.25
																36.1	4.23
																37.9	4.25

tive influence of duration and intensity of pre-adaptation on the dark adaptation process.

The data of Figs. 1 and 2 are in good general agreement with previous constant pupil measurements (Müller, 1931; Hecht, Haig, and Chase, 1937), but differ in exact detail, as is to be expected in view of the differences in experimental method, and in the sizes and positions of the retinal areas studied.

Comparison of Fig. 1 with Fig. 2 reveals that the effect of a rise in the degree of light adaptation, whether produced by increasing the intensity

of the light, or by lengthening the exposure, is to reduce the rate of rod dark adaptation. This change in rate has two components, a decrease in the slope of the function, and a displacement to the right on the time axis.

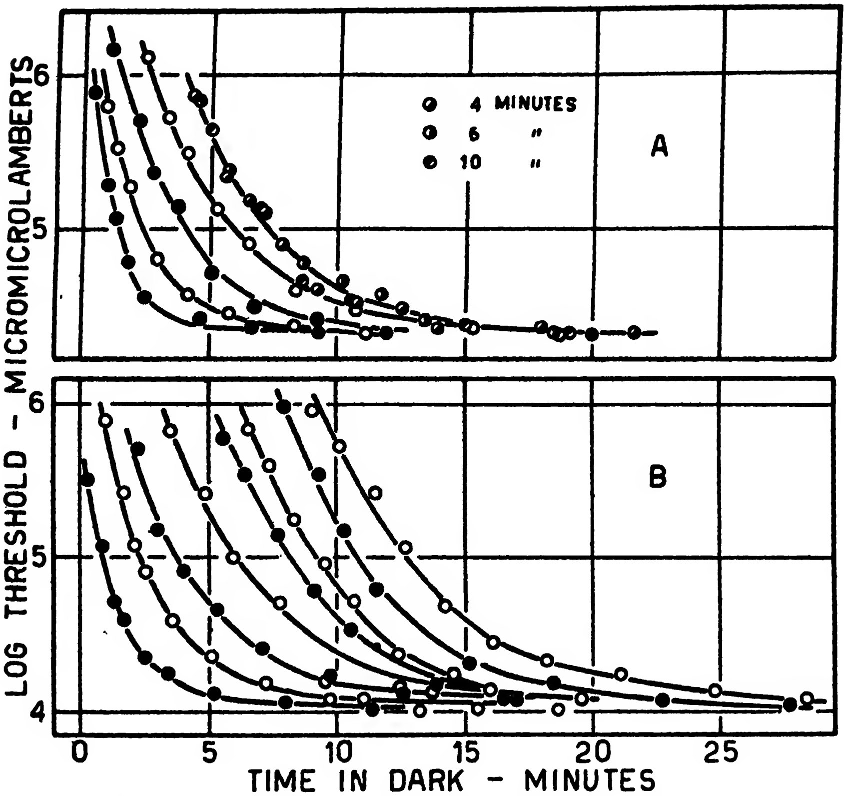

FIG. 1. Dark adaptation after different periods of exposure to light of 447 ml. intensity. Data in Tables I and II. Readings in which the test light appeared violet to the observer have been omitted. (*A*) Observer E. M. H., with 2 mm. artificial pupil. Duration of light adaptation from left to right in minutes: 0.1, 0.4, 1, 2, 4, 6, and 10. (*B*) Observer R. C., with Argyll Robertson pupil. Duration of light adaptation from left to right in minutes: 0.2, 0.5, 1, 1.2, 2, 5, 6, and 10.

The first effect is more marked after the lower degrees of light adaptation, and the second after the higher ones.

A critical point in the transition from high to low slopes appears at a pre-adapting intensity of about 40 ml. (400 photons[1]) in the data of the

[1] By definition, an external brightness of 1 ml. through a 2 mm. pupil illuminates the retina with a brightness of 10 photons, and through a 5 mm. pupil with a brightness of about 60 photons. However, Stiles and Crawford (1933) have shown that in practice this rule applies only to small pupillary diameters.

normal observer, and at about 100 ml. (*ca* 6000 photons) in those of observer R. C. This finding for the normal observer agrees very well with the estimate of 200 photons previously made (Hecht, Haig, and Chase, 1937)

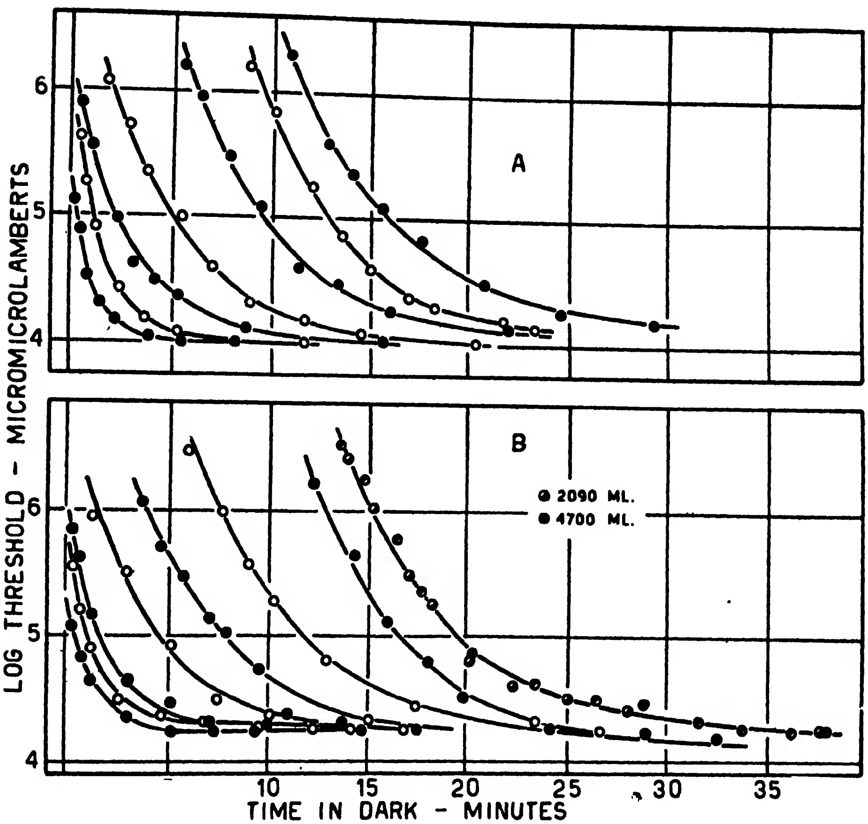

FIG. 2. Dark adaptation after 4 minute exposures to lights of different intensities. Data in Tables III and IV. Readings in which the test light appeared violet to the observer have been omitted. (*A*) Observer E. M. H., with 2 mm. artificial pupil. Intensity of light adaptation from left to right in millilamberts: 4, 20, 44, 447, 1150, 2090, and 4700. (*B*) Observer R. C., with Argyll Robertson pupil. Intensity of light adaptation from left to right in millilamberts: 4, 20, 44, 110, 200, 447, 1150, 2090, and 4700.

of the intensity of pre-adaptation at which the rapid type of dark adaptation is definitely established. For both subjects this transition is critical at a pre-adapting duration of about 1 minute.

Both displacement on the time axis and further changes in slope cease above a certain degree of light adaptation, which evidently is complete at

this point. An illustration of the latter is seen in Fig. 1*A*, the 6 and 10 minute points falling on the same curve with the 4 minute ones.[2] Similarly, in Fig. 2*B*, the 4700 ml. data coincide with the 2090 ml. ones.

IV

Displacement on the Time Axis

The quantitative aspects of these changes in the course of dark adaptation produced by varying the intensity and duration of the preceding light adaptation may be more adequately formulated by a graphical treatment of the positions and slopes of the curves.

For expressing the temporal positions of the curves, the number of minutes in the dark required for the threshold to descend to a given level above the final threshold, following the various pre-adapting intensities and durations, was obtained graphically from the data of Tables I-IV. Obviously it makes no essential difference in the result at what level above the final threshold this point is taken, provided it is such that every curve in both the intensity and duration series of each observer is accurately represented. The levels above the final thresholds selected were 0.75 log unit for observer R. C. and 0.70 log unit for observer E. M. H. The values so found (Table V) were plotted against the logarithms of the intensity (Fig. 3A) and the duration (Fig. 3B) of pre-adaptation.

The greater portions of the curves of Fig. 3 represent displacement of the dark adaptation curves on the time axis, only the segments representing pre-adaptations below about 100 ml. (R. C.), 40 ml. (E. M. H.), and 1 minute (both observers) having any considerable slope component. The curves in Fig. 3 are all sigmoid in form, but attain different heights and flatten out at the top to different degrees and at different levels. The slopes of the intensity and duration curves for the same observer are practically identical at comparable levels, the duration curves diverging from parallelism only at the upper ends.

[2] This means that light adaptation to a light of 447 ml. brightness through a 2 mm. pupil was complete in the normal observer within 4 minutes. It will be noted (Fig. 1 B), that in the case of observer R. C., light adaptation to a light of this brightness through a 5 mm. pupil was still incomplete after 10 minutes. Thus, even though the intensity of light adaptation was greater by a theoretical factor of more than 6 in the case of the observer with cirrhosis of the liver, its rate was much slower than in the case of the normal observer. Measurements of the rate of light adaptation of two other patients with liver cirrhosis (also having delayed dark adaptation) showed a similar delay. In all three subjects the speed of both functions was increased by vitamin A therapy (Haig, 1940). These observations clearly suggest that the rates of light adaptation and dark adaptation are governed by a common factor, presumably a catalyst.

TABLE V

Relation between duration and intensity of light adaptation, the time of appearance of a specific threshold, and the slope of the curve of dark adaptation. The specific thresholds, and the method of computing the slope are given in the text.

Observer	Adaptation duration	Time to specific threshold	Slope	Adaptation intensity	Time to specific threshold	Slope
	min.	*min.*		*millilamberts*	*min.*	
E. M. H.	0.1	1.4	1.77	4	0.6	2.16
	0.4	2.3	1.03	20	1.6	1.38
	1.0	4.0	0.65	44	3.1	0.65
	2.0	5.7	0.55	447	6.5	0.53
	4.0	7.1	0.55	1150	11.1	0.43
	6.0	7.1	0.55	2090	14.4	0.45
	10.0	7.1	0.55	4700	18.1	0.30
R. C.	0.2	1.2	1.18	4	0.4	1.54
	0.5	3.0	0.69	20	1.0	1.18
	1.0	4.8	0.51	44	1.7	0.89
	1.2	7.2	0.50	110	4.5	0.49
	2.0	9.3	0.49	200	7.7	0.44
	5.0	10.5	0.47	447	11.5	0.38
	6.0	12.0	0.45	1150	16.5	0.43
	10.0	13.9	0.38	2090	19.3	0.36
				4700	19.3	0.36

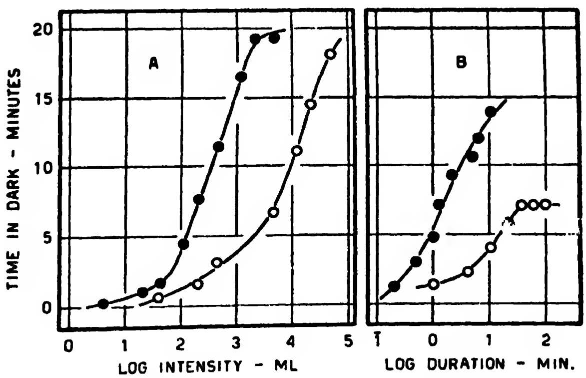

FIG. 3. Relation of the intensity (*A*) and the duration (*B*) of light adaptation to the time required for the dark adaptation function to attain a specific threshold. Data in Table V. The values on the abscissae refer to the data of R. C. (filled circles), those of observer E. M. H. (open circles) being displaced 1 logarithmic unit to the right.

The relatively low level at which the duration data of observer E. M. H. (Fig. 3B, open circles) reach a maximum constant value is obviously due to the low intensity of pre-adaptation employed in the duration experiments. With higher adapting intensities, greater displacements of the

dark adaptation function would be produced, and the completion of light adaptation would take place at higher displacement levels. Thus, the higher the intensity of pre-adaptation employed in the duration series, the more the displacement function of the latter should resemble that of the intensity series.

These comparisons reveal a striking similarity between the effects of intensity and duration of pre-adaptation upon the displacement in time of the dark adaptation function.

The significance of the component of the curves of Fig. 3 representing simple displacement in time, as distinguished from the small additional component attributable to changes in slope, is fairly well understood (Hecht, Haig, and Chase, 1937), and is predictable from mass law considerations (Winsor and Clark, 1936). Blanchard (1918) has observed that increasing the intensity of the pre-adapting light causes a rise in the instantaneous threshold in the dark. Since the same effect is no doubt also produced, within limits, by increasing the duration of light adaptation, it may be said that the higher the degree of pre-adaptation, the higher will be the initial dark adaptation threshold, and the farther the threshold will have to drop to attain the equilibrium level. Thus, the higher the degree of light adaptation, the longer will be the time required by the dark adaptation process to reach any given threshold value.

V

Change of Slope

For convenient graphical representation, the slope of the dark adaptation curves may be defined as the reciprocal of the time in the dark required for the threshold to descend 0.4 logarithmic unit at a given level above the final threshold.

On this basis, we have calculated the slopes at two levels of the dark adaptation function, a higher level, and a contiguous lower one. The two slopes were then averaged to obtain an over-all index of the slope of the function through a drop in threshold of 0.8 log unit. For observer R. C. the threshold intervals employed were from 1.15 to 0.75 log units, and from 0.75 to 0.35 log units above the final threshold. For observer E. M. H. the intervals were from 1.19 to 0.79, and from 0.79 to 0.39 log units above the final threshold.

The average slopes so computed are given in Table V, and have been plotted against the logarithms of the corresponding durations and intensities of light adaptation, as shown in Fig. 4.

Again in this figure, as in Fig. 3, the slopes of the intensity and of the duration series for the same observer are seen to be very nearly parallel. Thus, for both displacement on the time axis and for alteration of the slope of the dark adaptation function, the effects of intensity and of duration of pre-adaptation are found to be amazingly alike.[3]

The phenomenon of the decreasing slope of the dark adaptation function accompanying increases in the intensity of light adaptation was first reported for the retina as a whole by Blanchard (1918). It was later observed in the fovea by Fedorova (1927), and by Johannsen (1934). Since an analogous effect has been found in enzyme systems *in vitro*, *e. g.* in the auto-

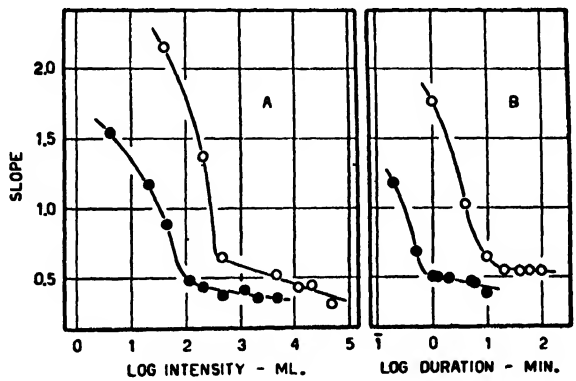

FIG. 4. Relation of the intensity (A) and the duration (B) of light adaptation to the slope of the dark adaptation function. Data in Table V. The values on the abscissae refer to the data of observer R. C. (filled circles), those of observer E. M. H. (open circles) being displaced 1 logarithmic unit to the right.

catalytic formation of trypsin from trypsinogen (Kunitz, 1939), it is evidently not a unique property of the visual mechanism, but, on the contrary, may have quite general chemical significance.

VI

Similarities in the Effects of Intensity and Duration

In view of the extraordinary conformities already noted in the effects of intensity and of duration of pre-adaptation on the course of dark adapta-

[3] It should perhaps be mentioned that the curves drawn through the data of Figs. 1 and 2 were computed from the relation $Kt = C - \log (\log I/\log I_f - 1)$, where I is the threshold intensity at time t in the dark, I_f the final threshold intensity, K the slope of the function, and C the intercept. It is of interest that the values of C for the data of Figs. 1 and 2 vary with the degree of light adaptation in much the same manner as do the displacement values of Fig. 3, while those of K vary as do the slopes plotted in Fig. 4.

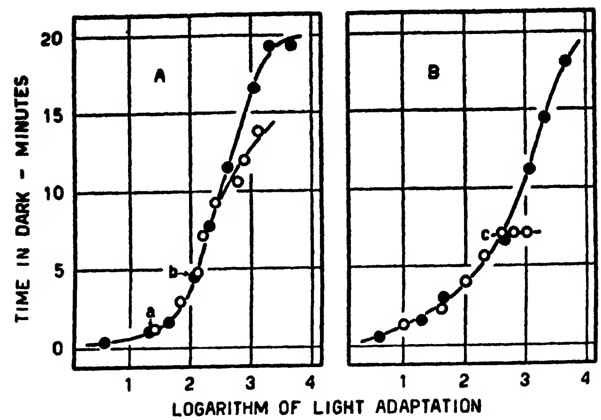

FIG. 5. Comparison of the influence of intensity (filled circles) and duration (open circles) of light adaptation upon the time required for the dark adaptation function to attain a specific threshold. (*A*) Observer R. C., (*B*) observer E. M. H. The values on the abscissae refer to the adapting intensity, those of the adapting duration having been moved up 2.1 log units (R. C.), and 2.0 log units (E. M. H.).

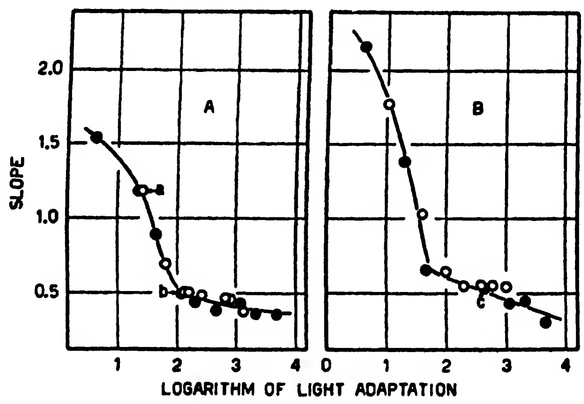

FIG. 6. Comparison of the influence of intensity (filled circles) and duration (open circles) of light adaptation upon the slope of the dark adaptation function. (*A*) Observer R. C., (*B*) observer E. M. H. The values on the abscissae refer to the adapting intensity, those of the adapting duration having been moved up 2.1 log units (R. C.), and 2.0 log units (E. M. H.). *a*, *b*, and *c*, indicate duration and intensity points which coincide in both Figs. 5 and 6.

tion, it is hardly surprising to discover that the intensity and the duration curves for both the displacement and the slope functions are superimposable over a considerable range by a shift along the abscissae. The amount of shift is identical for both functions, and very nearly the same for both observers. It is 2.10 logarithmic units for observer R. C., and 2.00 loga-

rithmic units for observer E. M. H. Figs. 5 and 6 show the results when the duration data are shifted to the right on the axis of abscissae by these amounts.

The figures show that changes in the duration or in the intensity of pre-adaptation produce the same amount of displacement on the time axis and the same degree of change of slope in the dark adaptation function over very wide limits. For observer R. C. these limits are approximately 20–200 ml. and 0.2–2.5 minutes, and for observer E. M. H. they are approximately 11–450 ml. and 0.1–4 minutes. For observer E. M. H. the correlation is almost perfect up to the point of complete light adaptation in the duration series, while for observer R. C., the correlation breaks down somewhat below this point.

Two pairs of points (*a* and *b* in Figs. 5 and 6) in the data of R. C., and one pair (*c* in Figs. 5 and 6) in those of E. M. H., happen to coincide almost exactly in both figures. Since this means that the dark adaptation curves which these pairs of points represent have the same position on the time axis and the same slope, they must be identical curves.

Both curves of the pair designated *c* are resultants of the same pre-adapting conditions (4 min. × 447 ml.), and hence their falling together in Figs. 5 and 6 merely serves to demonstrate the precision of the measurements and of the graphical analysis. Pairs *a* and *b*, however, are each resultants of widely different pre-adapting conditions, *a* representing 4 minutes × 20 ml. = 80 ml. minutes *versus* 0.2 minute × 447 ml. = 89 ml. minutes, and *b* representing 4 minutes × 110 ml. = 440 ml. minutes *versus* 1 minute × 447 ml. = 447 ml. minutes. The four sets of data have been plotted together, with appropriate adjustments of the threshold levels, in Fig. 7. It is clear from the figure that in each case the data of the intensity series coincide with those of the duration series.

The fact that the quantities of pre-adapting light for each pair of curves shown in Fig. 7 are almost identical is obviously an expression of the Bunsen-Roscoe law, which evidently holds for the entire range over which the displacement and slope functions of Figs. 5 and 6 are superimposable. This law states that for a given photolytic effect, duration and intensity of exposure to light bear a reciprocal relation; *i.e.*, $I \times t$ = a constant (Bunsen and Roscoe, 1862).

It is clear that for reversible photochemical systems, the upper limit of the exposure time for which the Bunsen-Roscoe relation holds is determined by the rate of the reverse dark process. The slower the dark process, the longer the exposure period may be without causing an appreciable departure from the reciprocal relation between time and intensity. Since the present

data conform to the Bunsen-Roscoe law up to pre-exposure durations of as long as 4 minutes, it must be concluded that the dark process during pre-exposure to the light intensities employed is exceedingly slow. Thus, the slowing down of dark adaptation as the degree of light adaptation is increased most likely constitutes the basis for the close adherence of the data to the Bunsen-Roscoe law.

In the present experiments all but one (0.1 ml., or 1 photon) of the pre-adapting intensities are above the intensity of less than 1 photon found to be necessary for maximum effectiveness of the rods in all other visual func-

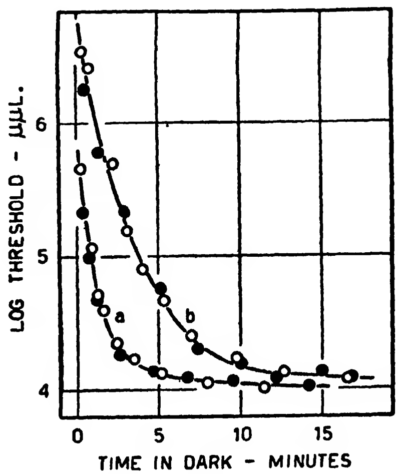

FIG. 7. The dark adaptation measurements represented by the pairs of coinciding points designated *a* and *b* in Figs. 5 and 6 plotted on the same coordinates. Light adaptation: (*a*) filled circles, 4 minutes × 20 ml.; open circles, 0.2 minute × 447 ml. (*b*) filled circles, 4 minutes × 110 ml.; open circles, 1 minute × 447 ml.

tions that have been measured (Hecht, 1934). Measurements of dark adaptation following these intensities of pre-adaptation may therefore be expected to reveal properties of the visual mechanism that are not disclosed by measurements of the dark-adapted threshold or of such functions as visual acuity, intensity discrimination, or critical fusion frequency.

Thus, for the threshold of the dark-adapted eye, the relation $I \times t = C$ has been shown (Piéron, 1920) to hold for exposure periods of no longer than 0.07 second, whereas for the light adaptation preceding dark adaptation, the present data indicate that this relation holds for exposure periods of as long as 4 minutes.

The reason for the decrease in slope of the dark adaptation function with increasing degrees of pre-adaptation remains obscure. Kühne (1879) has

found, and Wald (1935 *a, b*) confirmed, that, after bleaching, visual purple regenerates in excised retinas by two different routes, rapidly from its immediate photoproducts, and more slowly from its remote precursors. On the assumption that the retina *in situ* possesses a similar mechanism, it has been proposed (Winsor and Clark, 1936; Wald and Clark, 1937; Hecht, Haig, and Chase, 1937) that the dark adaptation process is compounded of two concomitant reactions, a fast and a slow one, the slope of the dark adaptation function depending upon the proportion in which the two reactions contribute to the total effect. In general outline, some such explanation of the variable slopes of the dark adaptation function seems not improbable.

Wald and Clark (1937), however, have attempted a more precise formulation of this comparison between the chemical findings in the retina and the results of dark adaptation measurements. They deduce that the classical visual cycle, as modified by Wald (1935 *a, b*), behaves in such a manner that in dark adaptation measurements, recovery from very short exposures to lights of high intensity will be found to be faster than recovery from prolonged exposures to lights of lower intensity, even though the degree of light adaptation is the same in the two cases. They have published dark adaptation data which seem to confirm this prediction (Wald and Clark, 1937).

The present results, indicating that duration and intensity of pre-exposure have, within wide limits, identical effects upon the course of dark adaptation, are at complete variance with the findings of Wald and Clark. Believing it possible that the effect which they observed may occur under more extreme conditions of pre-adaptation than those we had used, we have experimented with pre-exposure intensities and durations over the entire range within the limitations of our equipment. In none of these trials was the Wald-Clark effect observed. On the contrary, it was found that for equal degrees of light adaptation, as indicated by identical initial dark adaptation thresholds, the course of dark adaptation is the same whether pre-exposure is for a brief period to a light of high intensity or a prolonged period to a light of lower intensity. This rule applies even beyond the range in which the Bunsen-Roscoe law holds.

Two such experiments are illustrated graphically in Fig. 8. To insure physiological constancy, the long and the short exposure runs were made in immediate succession. Three sets of measurements were obtained following each species of pre-adaptation. It is observed (Fig. 8) that the quantities of pre-adapting light yielding identical dark adaptation curves in these experiments were not equal, being about twice as great for the prolonged

exposures as for the brief exposures. Considering the extreme range of the pre-adapting conditions, this is a surprisingly small departure from the Bunsen-Roscoe relation.

In view of the evidence (Reeves, 1918; Stiles and Crawford, 1933) that the early portions of dark adaptation measurements are likely to be influenced by dilatation of the natural pupil, it is possible that the differences between the present findings and those of Wald and Clark are attributable to the fact that in our experiments the pupillary diameter was held constant, while in theirs it was not.

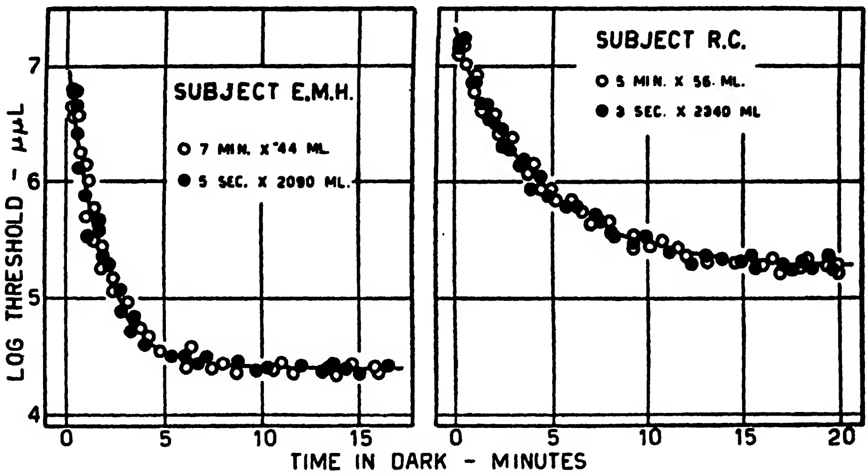

FIG. 8. Duration *versus* intensity of light adaptation. Each point is a single observation. Subject E. M. H., violet test light. Subject R. C., white test light. Adapting intensities and durations, as indicated.

SUMMARY

An increase in the degree of light adaptation causes a decrease in the slope of the subsequent rod dark adaptation function and a displacement of the function to the right on the time axis.

Over a wide range, these changes occur to the same extent whether the increase in the degree of light adaptation is produced by raising the intensity or by prolonging the exposure. Within these limits, the Bunsen-Roscoe reciprocity law applies to the intensity and duration of pre-exposure.

Over a still wider range, dark adaptation has the same course following brief exposure to a bright light as it has following prolonged exposure to a dim light, provided the degree of light adaptation is the same in both instances (as indicated by identical initial dark adaptation thresholds).

The author was assisted by Eleanor Mancell Haig.

BIBLIOGRAPHY

Blanchard, J., The brightness sensibility of the retina, *Phys. Rev.*, 1918, **11**, series 2, 81.

Bunsen, R., and Roscoe, H., Photochemische Untersuchungen: IV, *Ann. Physik*, 1862, **117**, 529.

Charpentier, A., L'inertie retinienne et la theorie des perceptions visuelles, *Arch. Ophth.*, 1886, **6**, 114.

Fedorova, V., *J. physique appliquée*, 1927, **4**, 67 (Russian). (Cited by Lasareff, P., Théorie ionique de l'excitation des tissues vivants, Paris, Blanchard, 1928, 240 pp.)

Haig, C., The influence of the pupillary light reflex upon dark adaptation measurements, *Anat. Rec.*, 1938, **72**, suppl., 82.

Haig, C., Dark adaptation measurements with natural and constant pupillary apertures, *Am. J. Physiol.*, 1939, **126**, 518.

Haig, C., Vitamin A and the rates of adaptation of the eye to light and darkness, *Anat. Rec.*, 1940, **78**, suppl., 163.

Haig, C., Hecht, S., and Patek, A. J., Jr., Vitamin A and rod-cone dark adaptation in cirrhosis of the liver, *Science*, 1938, **87**, 534.

Hecht, S., The nature of the photoreceptor process, in Murchison, C., A handbook of general experimental psychology, Worcester, Clark University Press, 1934, 704.

Hecht, S., Haig, C., and Chase, A. M., The influence of light adaptation on subsequent dark adaptation of the eye, *J. Gen. Physiol.*, 1937, **20**, 831.

Hecht, S., and Shlaer, S., An adaptometer for measuring human dark adaptation, *J. Opt. Soc. America*, 1938, **28**, 534.

Johannsen, D. E., The duration and intensity of the exposure light as factors in determining the course of the subsequent dark adaptation, *J. Gen. Psychol.*, 1934, **10**, 4.

Kroh, O., Die Weissempfindung der Stäbchenauges, *Z. Sinnesphysiol.*, 1922, **53**, 187.

Kroh, O., Über einen Fall von anomaler Functionsweise des Stäbchenapparats, *Z. Sinnesphysiol.*, 1922, **53**, 197.

Kühne, W., Chemische Vorgänge in der Netzhaut, in Herman's Handbuch der Physiologie, Leipzig, Vogel, 1879, **3**, pt. 1, 235.

Kunitz, M., Effect of the formation of inert protein on the kinetics of the autocatalytic formation of trypsin from trypsinogen, *J. Gen. Physiol.*, 1939, **22**, 293.

Müller, H. K., Über den Einfluss verschieden langer Vorbelichtung auf die Dunkeladaptation und auf die Fehlergrösse der Schwellenreiz-bestimmung während der Dunkelanpassung, *Arch. Ophth.*, Berlin, 1931, **125**, 624.

Patek, A. J., Jr., and Haig, C., The occurrence of abnormal dark adaptation and its relation to vitamin A metabolism in patients with cirrhosis of the liver, *J. Clin. Inv.*, 1939, **18**, 609.

Piéron, H., De la variation de l'énergie liminaire en fonction de la durée d'excitation pour la vision périphérique, *Compt. rend. Acad. Sc.*, 1920, **170**, 1203.

Reeves, P., The rate of pupillary dilation and contraction, *Psychol. Rev.*, 1918, **25**, 330.

Stiles, W. S., and Crawford, B. H., The luminous efficiency of rays entering the eye pupil at different points, *Proc. Roy. Soc. London, Series B*, 1933, **112**, 428.

Wald, G., Vitamin A in eye tissue, *J. Gen. Physiol.*, 1935 *a*, **18**, 905.

Wald, G., Carotenoids and the visual cycle, *J. Gen. Physiol.*, 1935 *b*, **19**, 351.

Wald, G., and Clark, A. B., Visual adaptation and chemistry of the rods, *J. Gen. Physiol.*, 1937, **21**, 93.

Winsor, C. P., and Clark, A. B., Dark adaptation after varying degrees of light adaptation, *Proc. Nat. Acad. Sc.*, 1936, **22**, 400.

THE EFFECT OF DETERGENTS ON THE CHLOROPHYLL-PROTEIN COMPOUND OF SPINACH AS STUDIED IN THE ULTRACENTRIFUGE*

By EMIL L. SMITH** AND EDWARD G. PICKELS

(From the Laboratory of Biophysics, Columbia University, and the Laboratories of the International Health Division of The Rockefeller Foundation, New York)

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I

INTRODUCTION

It has been demonstrated that in the green leaf chlorophyll is combined with protein by true chemical linkage. The chlorophyll-protein compound of the spinach leaf is insoluble in water and buffer solutions, but can be dispersed by detergents yielding brilliantly clear solutions. In addition to clarifying these solutions, the detergents denature the protein and change the absorption spectrum and other properties (Smith, 1941 *a*, *b*). Observations of the action of various detergents on the chlorophyll-protein compound have now been made using the ultracentrifuge.

II

Materials and Methods

All of the observations were made with fresh extracts of spinach leaves obtained by grinding mechanically in a porcelain mortar with sand and buffer solutions. Most of the sand and debris were removed by squeezing through muslin, and the extract was then centrifuged in a cold room at 1800–2000 R.P.M. The extracts were used within 1 or 2 days, and from the time of preparation until use were kept cold.

For most of the work the preparation was not further purified. In one instance, an extract was concentrated by centrifuging at 8000 R.P.M. in an air-turbine concentration centrifuge. The supernatant fluid containing the soluble proteins of the leaf extract was discarded, and the green pellets rubbed up in 0.2 M Na_2HPO_4. This preparation was used for three runs.

Solutions of the detergents were added immediately before beginning the measurements. Four detergents were used: digitonin obtained as crystalline digitalin from Eimer and Amend, New York; sodium desoxycholate obtained from Riedel de Haen,

* A preliminary report of this work was presented to the American Physiological Society in March, 1940 (Smith, 1940).

** John Simon Guggenheim Memorial Fellow (1938–1940).

New York; bile salts, a colorless crystalline preparation prepared from commercial sodium glycocholate in the Laboratory of Biophysics; and sodium dodecyl sulfate, a crystalline preparation obtained from Imperial Chemical Industries by Professor Keilin of the Molteno Institute, Cambridge, whom we wish to thank for a supply of this substance.

The air-driven vacuum ultracentrifuge described by Bauer and Pickels (1937) was used in these studies. The solutions were observed with the optical absorption method of Svedberg, and simultaneously with an automatic refractive index system designed especially for the ultracentrifuge and utilizing a scanning system similar to that described by Longsworth (1939) for electrophoresis measurements. Sedimenting solutions were photographed alternately with red and with violet light. For red light the Wratten "monochromat" filter No. 70 was used, and for the violet, a 1.5 mm. No. 511 Corning filter together with a 3 mm. No. 038 Corning filter. A 100 watt projection lamp was the source. The runs were made at a speed of 46,800 R.P.M. at temperatures near 25°C.

III

EXPERIMENTAL

Absorption measurements were carried out with both red and violet light in order to ascertain whether the carotenoid pigments sedimented differently from the chlorophyll. One difficulty that was encountered with all of the measurements was that the protein concentration could not be made very high. The relatively high concentration of chlorophyll present in the chloroplast complex, 7.9 per cent or 16 per cent in relation to the protein alone (Smith, 1941 *a*), together with the high extinction coefficient of the chlorophyll necessitated keeping the total concentration of protein low in order not to make excessively long exposures both for the absorption and refractive index readings. For this reason, the refractive index diagrams usually showed only protein boundaries of low concentration. Nevertheless, in order to obtain refractive index diagrams simultaneously, the pigment concentration had to be kept too high to give ideal conditions for absorption measurements. The sedimentation constants recorded were those obtained by the refractive index method.

A few runs were made with untreated leaf extracts. It was found that the total pigment sedimented at very low speeds, 2500–3000 R.P.M., and showed a purely random spread of particle sizes. This confirms the fact that the chlorophyll-protein complex of the spinach leaf is not in true solution. However, Price and Wyckoff (1938) and Loring, Osborn, and Wyckoff (1938) have reported obtaining clear green solutions from various leaves which show sharp boundaries with high but variable sedimentation constants.

1. Centrifugation of Detergents.—The experiments of McBain and his collaborators (McBain and Salmon, 1920) demonstrated that micelles are

formed in aqueous solutions of electrolytes such as soaps and other paraffin-chain salts. Sedimentation runs were therefore made with solutions of the four detergents used in these experiments. The results have already been reported elsewhere (Smith and Pickels, 1940). It was found that the non-electrolyte digitonin forms large micelles of homogeneous size in aqueous solution ($S_{20} = 5.9 \times 10^{-13}$). With the other detergents, sodium desoxycholate, bile salts, and sodium dodecyl sulfate (SDS), no detectable quantities of large micelles were found. The refractive index curves were characteristic of low molecular weight substances, there being no boundaries but only a relative decrease of concentration in the upper part of the sedimenting solution and some increase in the lower section. In the experiments with the leaf extracts, care was taken to distinguish between protein behavior and effects caused by the detergents alone.

2. *Effect of Digitonin.*—Chlorophyll-protein extracts in the presence of digitonin show a regularly sedimenting boundary which is equivalent in red and violet light. By comparison with the refractive index diagrams it was found that these absorption boundaries are identical with the refractive index boundary of the digitonin micelle. Thus, these boundaries are due not to the protein but to the digitonin micelle.[1] The refractive index pictures also show an additional boundary which is not represented in the absorption pictures; this boundary possesses an average sedimentation constant of 13.5×10^{-13} (Table I).

These experiments show that the pigments are detached from the protein, and are held in solution by the hydrophobic portion of the digitonin micelle. It does not seem likely that the chlorophyll remains attached to any very high molecular weight fragments since the sedimentation constant of the digitonin micelle is unaffected by the presence of the pigment. Yet the fact that no loss of pigment occurs on prolonged dialysis of a digitonin-treated solution (Smith, 1938) suggests that the pigment is still combined with particles too large to pass through a cellophane membrane.

Sedimentation of a digitonin-treated solution after prolonged dialysis indicates that some recombination of pigment and protein occurred. Comparison of absorption and refractive index diagrams shows that the pigment and protein sedimented together, but the material was quite inhomogeneous. Nevertheless, the bulk of this material did sediment at somewhat higher rates than that found for the dissociated protein particles. The refractive index diagram gave no trace of the digitonin boundary.

[1] A few preliminary runs using the light absorption method alone erroneously led us to attribute this boundary to the protein (Smith, 1938). These runs show the sedimentation constant characteristic of the digitonin micelle. One experiment which showed a double boundary was apparently due to an artefact.

When digitonin solution was added to the dialyzed preparation, the pigment was again dissociated from the protein and gave a sharply sedimenting boundary together with the digitonin micelle. The protein boundary observed on the refractive index diagram gave a sedimentation constant in line with values previously obtained. Fig. 1 shows for comparison the

TABLE I

Sedimentation Experiments with the Chlorophyll-Protein Compound in Different Detergents

Sedimentation constants were obtained from refractive-index boundaries. All runs were made at 46,800 R.P.M., equivalent to an average centrifugal force of 160,000 times gravity, at temperatures near 25°C. The optical thickness of the cell was 3 mm. Most of the solutions were buffered with 0.1 M sodium phosphate; the two alkaline ones, 8.8–8.9, contained 0.1 M borate, and the one at pH 4.85, 0.1 M acetate. The three runs made with an extract concentrated in the air-turbine concentration centrifuge are marked "concentrated preparation."

Detergent	Concentration	pH	$S_{20} \times 10^{-13}$	Remarks
	per cent			
Digitonin	2.5	7.61	12.8	
	2.5	8.80	13.3	
	2.5	7.80	13.4	Concentrated preparation
	0.63	7.87	14.5	
	2.5	7.83	13.7	Digitonin added to solution after initial removal of digitonin by dialysis
			Average 13.5	
Sodium desoxycholate	0.5	7.59	13.8	
	0.25	7.60	13.8	
Bile salts	3.0	7.50	13.5	Concentrated preparation
Sodium dodecyl sulfate	0.25	8.89	2.53	Prosthetic group is chlorophyll, solution is green
	0.25	4.85	2.84	Prosthetic group is phaeophytin, solution is yellow
	0.25	7.52	2.32	Solution initially green, at end of run some phaeophytin formation
			Average 2.56	
	2.5	7.76	1.69	Concentrated preparation

absorption photographs obtained with *a*, the dialyzed preparation, and *b*, the same solution to which digitonin has been added. The data for these experiments are included in Table I.

3. Effect of Sodium Desoxycholate and Bile Salts.—With these solutions the absorption photographs show no sedimenting boundaries whatsoever, indicating that the chlorophyll is dissociated from the protein. The refractive index photographs clearly show only one sedimenting boundary

similar to that found with digitonin and possessing the same sedimentation constant (values in Table I). Digitonin and these two detergents therefore act in the same way on the chlorophyll-protein compound, dissociating the pigments from the protein and breaking the material into particles of the same size. In the experiment with bile salts using a concentrated

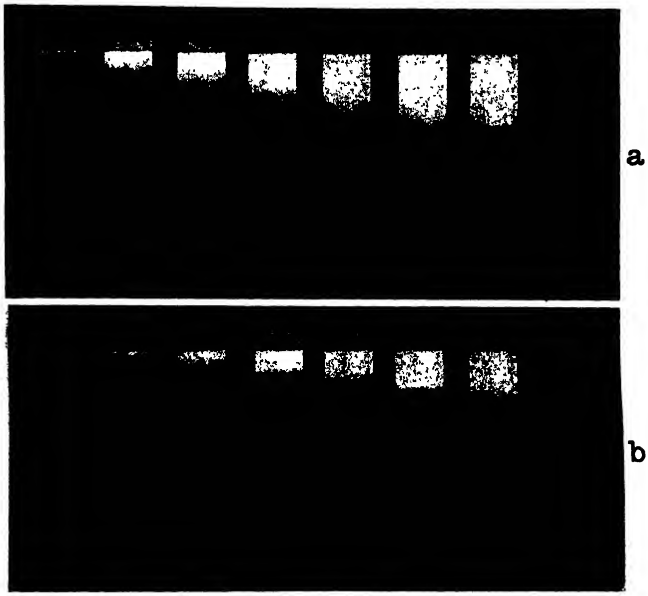

FIG. 1. Sedimentation photographs of *a*, a dialyzed digitonin-treated extract, and *b*, the same solution with digitonin again added. The dialyzed extract shows the inhomogeneous and comparatively rapid sedimentation of the pigment after removal of the digitonin. When digitonin was again added equivalent to the initial concentration (2.5 per cent), a sharply defined boundary (*b*) was obtained which was coincident with that of the colorless digitonin micelle ($S_{20} = 5.9 \times 10^{-13}$) observed refractometrically. This coincidence is also shown in the first absorption pictures by the presence of a dark band; this band corresponds to the micelle boundary which has caused a deviation of light beyond the aperture of the camera lens. Both sets of photographs were taken with red light at 10 minute intervals. The solutions contained 0.1 M phosphate at pH 7.8.

leaf extract, a large part of the protein sedimented irregularly and inhomogeneously. These particles had a wide variation in size; some were smaller and some larger than the principal component.

A single experiment with a concentrated urea solution as the solvent was performed using only the light absorption method. No sedimentation of the pigment occurred showing that in this solvent also, the chlorophyll was dissociated from the protein.

4. Effect of Sodium Dodecyl Sulfate.—It has already been shown (Smith, 1941 *b*) that sodium dodecyl sulfate (SDS) alters the nature of the prosthetic group of the chlorophyll-protein compound. In weakly acid solutions, magnesium is rapidly eliminated from the chlorophyll, converting it to phaeophytin; in alkaline solutions this reaction takes place very slowly. It was therefore of considerable interest to study the effect of pH on the sedimentation constant. Three runs were carried out in 0.25 per cent SDS,

FIG. 2. Absorption photographs taken alternately at 5 minute intervals with *a*, red, and *b*, violet, light, showing sedimentation of the chlorophyll-protein in 0.25 per cent sodium dodecyl sulfate at pH 8.9. No differences are detectable in the two regions of the spectrum. The common sedimenting boundary was coincident with the protein boundary ($S_{20} = 2.6 \times 10^{-13}$) detected by the refractive index method, showing attachment of the pigment to the protein.

at pH 4.85 where phaeophytin formation is complete in a few minutes, at pH 7.52 where the reaction requires many hours for half completion, and at pH 8.89 where only barely detectable changes take place in 24 hours. The results demonstrate that at all three pH values, the prosthetic group remains attached to the protein component. Fig. 2 shows absorption photographs taken alternately in red and in violet light at pH 8.89. The refractive index curves show only a single sedimenting boundary corresponding to the sedimentation of the pigment shown in the absorption pictures. The absorption photographs show that the single boundary is identical in

the two regions of the spectrum, and demonstrate that the carotenoid and chlorophyll pigments remain attached either to identical particles or to particles of the same size. The same sedimentation constant (Table I) was found at all three pH values; the average is 2.56×10^{-13}. It is apparent that the average size of the particles is not affected by the presence or absence of magnesium in the chlorophyll. The effect of SDS differs strikingly from the other detergents; not only does the prosthetic group remain attached to the protein, but the particles are split into apparently homogeneous fragments of lower sedimentation constant and are therefore of smaller size.

A single run was carried out in the presence of 2.5 per cent SDS using a more concentrated preparation of the chloroplast material. In the presence of the ten times higher concentration of SDS, the prosthetic group still remained attached to the protein, but the protein was further reduced in size yielding an S_{20} value of 1.69×10^{-13}. A photograph of one of the refractive index curves is shown in Fig. 3. Since the photographs were taken with visible light (mercury line of 546 mμ) the boundary on the refractive index curve shows the absorption of the pigments to correspond exactly with the schlieren pattern.

If it is assumed that the other physical characteristics of the particles and solvents are roughly the same for the two different concentrations of SDS, the approximate relationship between the size of the particles is as the ratio of the three-half powers of the sedimentation constants, or $S_1^{3/2}/S_2^{3/2} = M_1/M_2$. The ratio found was 0.54 using the average value of 2.56 for S_2 and 1.69 for S_1. The ratio suggests that at the higher SDS concentration, the particles are split into approximately half the size. A similar comparison between the sedimentation constant in digitonin ($S=$ 13.5) and in 0.25 per cent SDS, gives a size ratio of 12 to 1.

When the areas under the boundaries of the refractive index curves were measured, it was found that the apparent concentration of the sedimenting material in SDS was higher than that found by spectrophotometric measurement. The concentration of the chlorophyll-protein compound was estimated spectrophotometrically by measuring the height of the main absorption band in the red end of the spectrum. In the experiment with 2.5 per cent SDS using the values previously determined (Smith, 1941 *a*) from the average chlorophyll content of 7.9 per cent, the concentration of the chloroplast material was 1.3 per cent; using the chlorophyll content of 16 per cent in relation to the protein alone, the concentration was 0.63 per cent. Assuming the usual refractive index value for proteins, the concentration of sedimenting material was 2.4 per cent. The appreciably

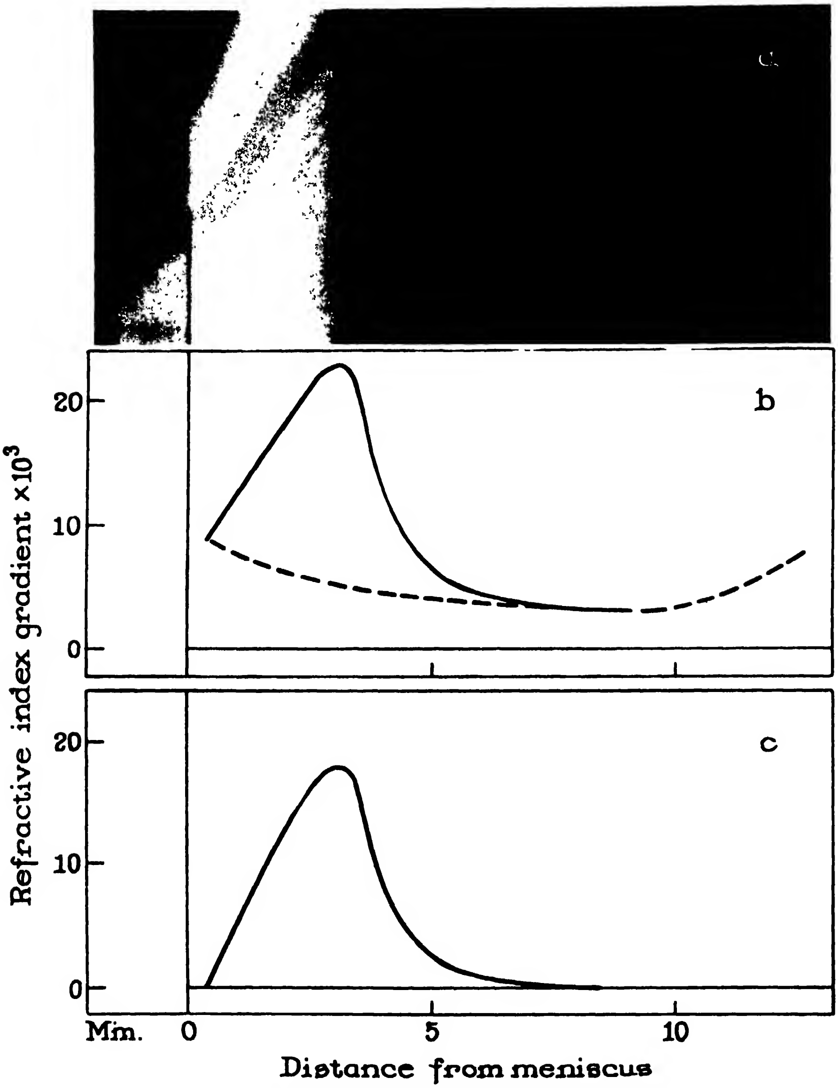

FIG. 3. Refractive index gradient curve, obtained after 3 hours, of a chlorophyll-protein solution in 2.5 per cent sodium dodecyl sulfate at pH 7.8. The sedimentation constant was approximately 1.7×10^{-13} cm./sec./dyne. A very high concentration of chlorophyll-protein was used to increase the size of the index curve. Although green light was used, a 15 minute exposure was necessary; this caused a reduction in the photographic definition of the curve *a*. That protein and pigment sedimented together is evident from the coincidence of the absorption and index boundaries *a*. The dotted baseline in *b* is the index curve for 2.5 per cent SDS alone. Curve *c* represents the difference between the dotted and smooth curves of *b*. Measurement of its area shows too high a concentration to be explained by the protein alone, indicating possible association with SDS. The unsymmetrical shape of the difference curve *c* is to be expected for very low sedimentation rates.

higher concentration of sedimenting material suggests that a considerable amount of SDS was sedimenting together with the protein, giving the effect of an increase in concentration of protein. Yet it is difficult to assess what effect this had on the apparent sedimentation constant. If it tends to increase the size of the sedimenting particles, the true size of the particles in 2.5 per cent SDS would be even smaller than half that in 0.25 per cent solution.

In one experiment with digitonin as the detergent and the same protein solution, the concentration of protein from the area of the refractive index curve was 0.7 per cent as compared with 1.3 and 0.63 per cent as estimated spectrophotometrically. Here there does not seem to be any appreciable quantity of detergent influencing the concentration of sedimenting material. This is to be expected since the total digitonin sediments as apparently homogeneous micelles.

IV

DISCUSSION

It has been known for some time that proteins can be split by various substances into fragments of lower molecular weight. The studies of Svedberg and his collaborators (Svedberg, 1937) have shown that acid or alkaline solutions cause the splitting of many proteins into particles smaller than those found over the range of pH near neutrality. Various proteins have been shown to be split by urea (Burk and Greenberg, 1930; Burk, 1937). Steinhardt (1938) has observed that while horse hemoglobin is split by concentrated urea solutions into halves, the absorption spectrum and oxygen binding capacity are not changed. The action of urea in splitting proteins is not unique and there are undoubtedly numerous reagents which will split proteins but these have been studied little as yet. Sreenivasaya and Pirie (1938) found that the tobacco mosaic virus is inactivated by SDS with the separation of nucleic acid and splitting of the protein into fragments. However, these investigators did not study the size of the split particles.

The action of the detergents studied in the present investigation shows that different reagents produce quite different effects on the same protein. SDS is unique among those studied since it is capable of converting chlorophyll into phaeophytin in neutral solutions, and it does not detach the prosthetic group from the protein. The other detergents attack different linkages since the chlorophyll is liberated or remains combined with only small fragments of the protein which are not sedimentable at the gravitational forces used. The latter possibility seems the more probable since pigment

is not lost on dialysis showing that the particles are still too large to pass through an ordinary cellophane membrane.

Not only do the various splitting agents produce different effects on the same protein but their action is likely to be different on other proteins. Urea, for example, does not affect the molecular weight or catalytic activity of pepsin (Steinhardt). With visual purple, a conjugated carotenoid-protein (Wald, 1935), the bleaching properties of the pigment are not affected by digitonin, bile salts, or sodium desoxycholate. However, after bleaching by light, regeneration *in vitro* takes place in the presence of the first two detergents but not in solutions of sodium desoxycholate (Chase and Smith, 1939). Moreover, dilute solutions of SDS instantaneously bleach visual purple in the dark.[2] It is clear that no rule can be laid down for the probable action of these detergents.

True molecular weight values for the chlorophyll-protein compound of spinach cannot be assessed as yet. The observations of Price and Wyckoff, and Loring, Osborn, and Wyckoff on the leaves of other plants suggest very high molecular weights of the same magnitude as those of the various hemocyanins. Since the protein of the spinach leaf is insoluble, no comparison is possible. The particle sizes found in the various detergents do suggest certain units which may be of importance. The sedimentation constant of 13.5×10^{-13} found in three detergents is equivalent to a molecular weight of at least 265,000. This molecular weight value is of the approximate order of magnitude previously found for many plant proteins such as phycocyanin, phycoerythrin, and many seed globulins (Svedberg, 1937). For the spinach leaf, 265,000 represents the minimum size of the protein in native form.

In all of the experiments, a careful examination was made for evidence of differential behavior of the various chloroplast pigments. No differences were found for chlorophylls *a* and *b*, and the carotenoids always followed the chlorophylls. This shows a strong association of all these pigments, and suggests that the carotenoids as well as the chlorophylls are chemically bound to the chloroplast protein.

SUMMARY

1. The chlorophyll-protein compound of the spinach leaf has been studied in the air-driven ultracentrifuge using the Svedberg light-absorption method, and a direct-reading refractive index method.

2. When the untreated extracts are centrifuged at low speeds, the green

[2] Unpublished observations by one of us (Smith, 1939).

protein sediments with a purely random spread of particle sizes confirming the fact that the protein is not in true solution.

3. In the presence of digitonin, bile salts, and sodium desoxycholate, the extracts are clarified. These detergents split the chlorophyll from the protein and the protein itself shows a sedimentation constant of 13.5×10^{-13} equivalent to a molecular weight of at least 265,000 as calculated from Stokes' law. This probably represents the minimum size of the protein in native form.

4. Sodium dodecyl sulfate, a detergent which also clarifies the leaf extracts, shows a different behavior. The prosthetic group remains attached to the protein but the protein is split into smaller units. In 0.25 per cent SDS, S_{20} is 2.6×10^{-13} over a pH range of 5 to 9, although at the acid pH chlorophyll is converted to phaeophytin. In 2.5 per cent SDS, S_{20} is 1.7×10^{-13} suggesting a further splitting of the protein.

5. No differences in behavior were found for the various chloroplast pigments.

BIBLIOGRAPHY

Bauer, J. H., and Pickels, E. G., An improved air-driven type of ultracentrifuge for molecular sedimentation, *J. Exp. Med.*, 1937, **65**, 565.

Burk, N. F., Osmotic pressure, molecular weight, and stability of amandin, excelsin, and certain other proteins, *J. Biol. Chem.*, 1937, **120**, 63.

Burk, N. F., and Greenberg, D. M., The physical chemistry of the proteins in non-aqueous and mixed solvents. I. The state of aggregation of certain proteins in urea-water solutions, *J. Biol. Chem.*, 1930, **87**, 197.

Chase, A. M., and Smith, E. L., The regeneration of visual purple in solution, *J. Gen. Physiol.*, 1939, **23**, 21.

Longsworth, L. G., A modification of the schlieren method for use in electrophoretic analysis, *J. Am. Chem. Soc.*, 1939, **61**, 529.

Loring, H. S., Osborn, H. T., and Wyckoff, R. W. G., Ultracentrifugal isolation of high molecular weight proteins from broad bean and pea plants, *Proc. Soc. Exp. Biol. and Med.*, 1938, **38**, 239.

McBain, J. W., and Salmon, C. S., Colloidal electrolytes. Soap solutions and their constitution, *J. Am. Chem. Soc.*, 1920, **42**, 426.

Price, W. C., and Wyckoff, R. W. G., The ultracentrifugation of the proteins of cucumber viruses 3 and 4, *Nature*, 1938, **141**, 685.

Smith, E. L., Solutions of chlorophyll-protein compounds (phyllochlorins) extracted from spinach, *Science*, 1938, **88**, 170.

Smith, E. L., An ultracentrifugal study of the action of some detergents on the chlorophyll-protein compound of spinach, *Am. J. Physiol.*, 1940, **129**, 466.

Smith, E. L., The chlorophyll-protein compound of the green leaf, *J. Gen. Physiol.*, 1941 *a*, **24**, 565.

Smith, E. L., The action of sodium dodecyl sulfate on the chlorophyll-protein compound of the spinach leaf, *J. Gen. Physiol.*, 1941 *b*, **24**, 583.

Smith, E. L., and Pickels, E. G., Micelle formation in aqueous solutions of digitonin, *Proc. Nat. Acad. Sc.*, 1940, **26**, 272.

Sreenivasaya, M., and Pirie, N. W., The disintegration of tobacco mosaic virus preparations with sodium dodecyl sulfate, *Biochem. J.*, London, 1938, **32**, 1707.

Steinhardt, J., Properties of hemoglobin and pepsin in solutions of urea and other amides, *J. Biol. Chem.*, 1938, **123**, 543.

Svedberg, T., Protein molecules, *Chem. Rev.*, 1937, **20**, 81.

Wald, G., Carotenoids and the visual cycle, *J. Gen. Physiol.*, 1935, **19**, 351.

FORMATION OF AUXIN IN YEAST CULTURES

By TRUE W. ROBINSON AND T. J. B. STIER

(From the Biological Laboratories, University of Illinois, Urbana, Illinois, and the Biological Laboratories, Harvard University, Cambridge, Massachusetts)

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The production of auxin by yeast cells resembles the formation observed in other organisms such as *Rhizopus* and *Rhizobium* (Thimann, 1939)[1] which "release" auxins into their culture medium. Thus, we have found far more auxin in the growth medium of yeast than Kögl and Kostermans[2] extracted from the cells. The amounts of auxin in the growth medium were found to vary considerably with the age of the culture, the rate of growth of the cells, and both the initial and final composition of the growth medium.

II

A pure strain of bakers' yeast, *Saccharomyces cerevisiae*, was grown under pure culture conditions in several modifications of Williams' medium.[3] The basic medium consisted of potassium dihydrogen phosphate 2.0 gm./l., ammonium sulfate 3.0 gm./l., magnesium sulfate 0.25 gm./l., calcium chloride 0.25 gm./l., asparagine 1.5 gm./l., and sucrose 20 gm./l. This medium was supplemented with the following initial concentrations of Bacto-peptone: 0, 0.01, 0.1, 1.0, 10 per cent. Samples were withdrawn at various intervals after seeding. On each of these, cell counts and auxin tests were made. Since approximately as much auxin was obtained by mixing the centrifuged but unextracted medium directly with agar as by extracting the centrifuged or non-centrifuged suspension with chloroform,[4] the former method was consistently used. The temperature was 25°C.; aerobic conditions were maintained in the culture towers by bubbling moistened, sterile air through the suspension.

The total auxin in the centrifuged media is plotted in Fig. 1 as a function of the age of the culture. The growth of the culture in 0.1 per cent peptone (dashed line) is also given. These two curves show a complete inverse relation between the auxin concentration and the rate of cell multiplication in the 0.1 per cent peptone medium throughout the experiment. This medium gave the highest auxin yield, namely, 450 auxin units per ml. at

[1] Thimann, K. V., *Tr. 3rd Commission Internat. Soc. Soil Sc.*, 1939, vol. A, 24.

[2] Kögl, F., and Kostermans, D. G. F. R., *Z. physiol. Chem.*, 1934, **228**, 113.

[3] Williams, R. J., *J. Biol. Chem.*, 1920, **42**, 259.

[4] Robinson, T. W., and Woodside, G. L., *J. Cell. and Comp. Physiol.*, 1937, **9**, 241.

225 hours after inoculation. At this time the 0.01 per cent peptone medium gave 113 units per ml., the 0 per cent peptone medium gave 35 units per ml., and the 1 and 10 per cent media gave only 6 units per ml. The auxin concentration was still rising in all media at 225 hours.

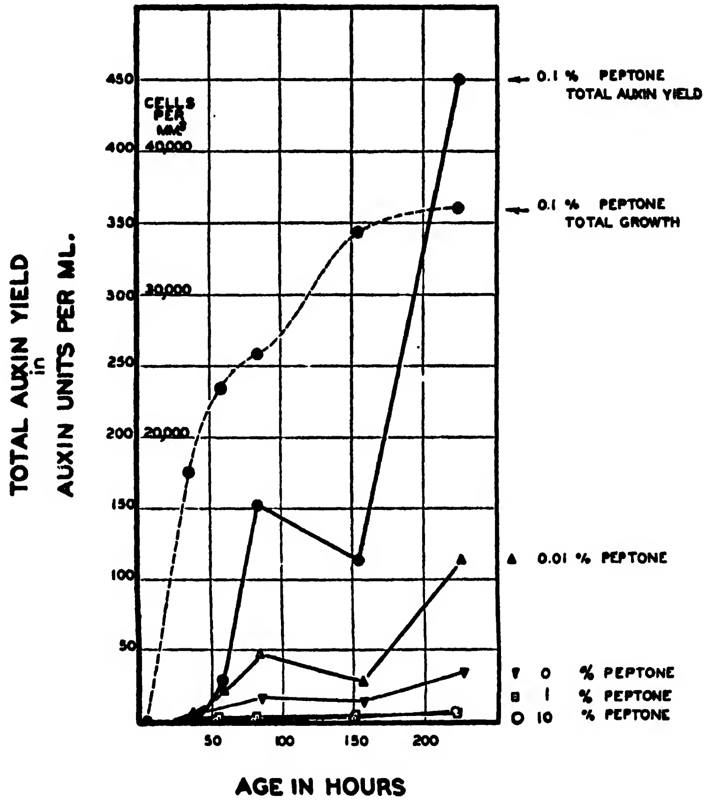

FIG. 1. The total auxin in centrifuged media from towers containing different amounts of Bacto-peptone is plotted as a function of the age of the culture at the time when a sample of the suspension was removed. The dashed line is the growth curve of the culture in 0.1 per cent peptone and can be compared with the total auxin yield from the same medium.

The greatest *rate* of auxin production (calculated as auxin units per cell per hour) was obtained in all media between 55 to 85 hours after inoculation. On the basis of 1 ml. of cell-free medium the rates were as follows: 0 per cent peptone, 890×10^{-6} auxin units per cell per hour; 0.01 per cent peptone, 790×10^{-6}; 0.1 per cent peptone, 190×10^{-6}; and the 1 and 10 per cent peptone less than 0.5×10^{-6}. In all these media during any given interval there was an inverse relation between the rate of auxin yield

and the rate of cell multiplication. With increasing peptone concentrations, the rate of cell multiplication increased, whereas the rate of auxin yield decreased. Note that the greatest rate of auxin production occurred in the basic medium free of peptone where there was practically no growth. Whether observed in different cultures during the same interval or in one culture at different intervals, the auxin yield seemed to be inversely correlated with cell multiplication.

In a second series of experiments, concentrations of 0.05, 0.1, 0.2, 0.5 per cent Bacto-peptone and 0.1 per cent Witte-peptone were tested. There

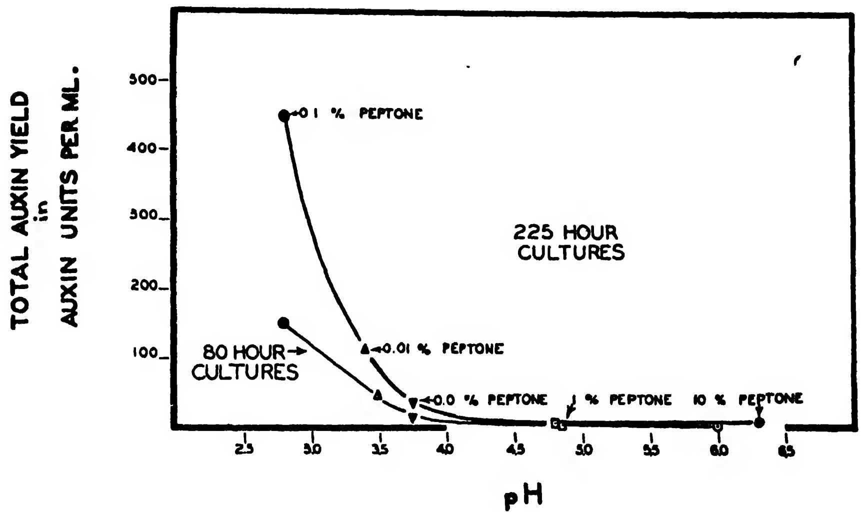

FIG. 2. Total auxin yield is plotted against pH of the same sample of centrifuged medium in which the auxin yield was tested. The relation after 80 hours and also after 225 hours of growth in the various peptone media are plotted.

was a low auxin yield in the 0.5 and 0.2 per cent Bacto-peptone media (3 and 10 units/ml.), a higher yield as before in the 0.1 per cent (90 units/ml.), a still higher yield in the 0.05 per cent (120 units/ml.). As in the first Bacto-peptone series, the rate of growth increased with the peptone concentration, while the auxin yield decreased. Much more auxin was obtained from the 0.1 per cent Witte-peptone medium than from the faster growing 0.1 per cent Bacto-peptone culture.

A correlation, apparent during most of the growth period between the total auxin yield and the pH of the centrifuged medium is shown in Fig. 2 for samples removed at 80 and 225 hours. Probably this correlation is not due to the hydrolysis in an acid medium of an inactive salt of auxin, since,

according to Dolk and Thimann,[5] heteroauxin, which appears to be active only in the undissociated acid form, is 50 per cent dissociated at a pH of 4.75. Had the auxin present in the medium behaved like heteroauxin, then 50 per cent of the active form, as determined by the coleoptile test, should have been obtained at a pH of 4.75. Yet even when the pH had decreased to 3.75, we obtained only 10 per cent of the total yield. The pH may be an important controlling factor in auxin production, however, for the production in our experiments occurred mainly below a pH of 4.5 and increased with higher hydrogen ion concentrations. In fact, if the total auxin yield at 225 hours is plotted not against pH but against the hydrogen ion concentration, a straight line with a slope of 2.8×10^{6} is obtained.

In testing the effect of sugar on the yield of auxin, towers were prepared with 0.1, 1.0, 2.0, 10.0, and 20.0 per cent sucrose. All contained 0.1 per cent Bacto-peptone and the usual basic Williams' medium. The results indicated that the auxin yield at any given time was proportional to the original concentration of sucrose in the various towers. Over 1000 auxin units per ml. of centrifuged medium were obtained from the 10 per cent sucrose medium. This is more than is normally found in mammalian[6, 7] urine, one of the richest known sources to date.

III

Of special interest in these investigations is the apparent effect of the auxin in the various peptone media on the size and shape of the yeast cells. In the media containing 0.2 per cent or more peptone and consequently having very low auxin concentrations, the cells were of normal size and shape (small and slightly elliptical); whereas, in the other media having high auxin concentrations the cells were large and sometimes elongated many times their normal diameter. This enlarged and elongate condition was only associated with those media containing considerable amounts of auxin and an initial concentration of 0.1 per cent or less Bacto-peptone. Somewhat similar modifications were found by Wyckoff and Luyet[8] and Oster[9] in plate cultures of yeast which had been irradiated with ultraviolet light; by Zahl, Koller, and Haskins[10] in *Aspergillus* spores similarly treated

[5] Dolk, H. E., and Thimann, K. V., *Proc. Nat. Acad. Sc.*, 1932, **18**, 30.

[6] Kögl, F., and Haagen Smit, A. J., *Proc. K. Akad. Wetensch. Amsterdam*, 1931, **34**, 1411.

[7] Kögl, F., Haagen Smit, A. J., and Erxleben, H., *Z. physiol. Chem.*, 1933, **214**, 241.

[8] Wyckoff, R. W. G., and Luyet, B. J., *Radiology*, 1931, **17**, 1171.

[9] Oster, R. H., *J. Gen. Physiol.*, 1934–35, **18**, 251.

[10] Zahl, P. A., Koller, L. R., and Haskins, C. P., *J. Gen. Physiol.*, 1938–39, **22**, 689.

with ultraviolet radiation, and by Richards[11] in liquid cultures of yeast incubated at 30°C. Whether the formation of these elongate giant cells is dependent upon the presence of a high concentration of auxin will be investigated presently.

IV

Recent work of Skoog and Thimann (1940)[12] on the liberation of auxin from plant tissues during extraction gives us a working hypothesis for future investigations. Their evidence indicates that the bulk of the auxin in cells is "bound to a protein." It seems reasonable, therefore, that certain growth conditions might favor the breakage of the link between auxin and its protein carrier and thus accelerate its "rate of formation," or better its rate of "excretion" into the medium. The excellent positive correlation obtained between pH concentration in the various media and the yield of auxin (see p. 768) illustrates the operation of this mechanism. These various findings now make it possible to separate clearly the two fundamental problems involved in studying auxin formation in cultures of microorganisms, namely, factors involved in its intracellular synthesis and factors involved in releasing the intracellular store of bound auxin.

SUMMARY

We have found far more auxin in the culture media of bakers' yeast than was obtained by Kögl and Kostermans from the cells themselves. The production of auxin by yeast cells resembles the formation observed in other organisms such as *Rhizopus* and *Rhizobium* which also form auxins in their culture media.

The auxin yield was found to increase with the concentration of sucrose and to decrease with the concentration of peptone. An inverse relation with the rate of cell multiplication was observed. Enlarged and elongated cells appeared only in those media which contained considerable amounts of auxin.

The total auxin yield in the various cultures was found to be directly proportional, below pH 5, to the hydrogen ion concentration. Thus, it was proposed that certain growth conditions favor the breakage of the link between auxin and its protein carrier (Skoog and Thimann, 1940) and consequently accelerate the rate of excretion of auxin into the growth medium.

[11] Richards, O. W., *J. Physic. Chem.*, 1928, **32**, 1865.

[12] Skoog, F., and Thimann, K. V., *Am. J. Bot.*, supplement, 1940, **27**, 19.

LONGITUDINAL IMPEDANCE OF THE SQUID GIANT AXON

By KENNETH S. COLE and RICHARD F. BAKER

(From the Department of Physiology, College of Physicians and Surgeons, Columbia University, New York, and the Marine Biological Laboratory, Woods Hole, Massachusetts)

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INTRODUCTION

Measurements of the transverse alternating current impedance of the squid giant axon have given a membrane capacity of about 1 microfarad/cm.^{2} (Curtis and Cole, 1938) and from longitudinal measurements of the direct current resistance of this axon, a membrane resistance of about one thousand ohms cm.^{2} has been found (Cole and Hodgkin, 1939). From these values the resistance and reactance components, R_s and X_s, of the membrane impedance may be calculated at various frequencies, and R_s and X_s, taken as abscissae and ordinates respectively, give the impedance locus of the membrane. This locus will be a circular arc (Cole, 1928, 1932) and, when the dielectric loss of the membrane is ignored, the locus will be the semi-circle of Fig. 1 *a*. For this simplified membrane the longitudinal impedance of the axon, as measured between two electrodes some distance apart along the length of the axon, may now be calculated by cable theory as shown below. In this manner a locus of the form shown in Fig. 1 *b* is predicted by equation (6) for the longitudinal impedance of a single axon having the membrane impedance locus of Fig. 1 *a*.

Longitudinal impedance data over a wide frequency range were first taken on the squid giant axon in connection with the direct current resistance experiments (Cole and Hodgkin, 1939) because high frequency measurements, in which the membrane impedance would be negligible, were needed to support the theory used to calculate the membrane resistance. A few sets of measurements over the complete frequency range were made primarily to determine the lowest frequency allowable for this purpose and incidentally to obtain data for comparison with the predicted theoretical impedance locus shown in Fig. 1 *b*. At frequencies above 500 cycles, the equivalent parallel capacity and resistance of the axons varied with frequency in approximately the predicted manner. At frequencies below 200 cycles, the resistance for one of the axons decreased below a maximum value

at about 200 cycles and for another axon the capacity actually went through zero. At 50 cycles a capacity of 0.01 μf had to be added to the *unknown* arm of the Wheatstone bridge to obtain a balance for this axon, as there was no provision in this equipment for measuring a net inductive reactance. All probable sources of error were found to be unimportant, and the completely unexpected presence of an inductance in the axon was apparently demonstrated. This inductance seemed to be associated with the membrane because the inductive reactance first decreased as the axon deteriorated and then was replaced by a capacitative reactance at all frequencies.

Further measurements were not possible at that time and the observations obviously needed to be confirmed and extended. All possible factors

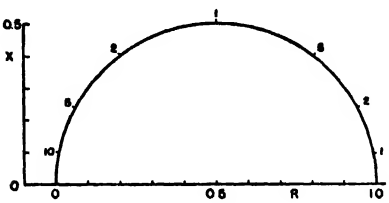

FIG. 1*a*

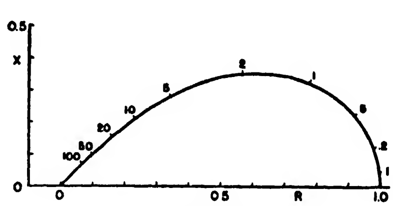

FIG. 1*b*

FIG. 1. Theoretical impedance loci, series resistance, R, *vs.* series reactance, X. (*a*) Locus for a membrane having a leakage resistance in parallel with a loss-free capacity. (*b*) Longitudinal locus, as given by equation (6), for an axon with membrane properties shown in (*a*). Frequencies are indicated in terms of the characteristic frequency of the membrane.

outside of the axon, from the apparatus to the oil which surrounded the interpolar stretch of the axon, should be eliminated. Then direct evidence should be obtained to localize the inductive structure in the connective tissue, the membrane, or the axoplasm. And finally, as much information as possible should be obtained about this structure and if, as suspected, the membrane was responsible, the relation of the inductance to the capacity and conductance should be determined. This program was undertaken in the summers of 1939 and 1940, and, although it is not satisfactorily completed, the results themselves are certainly indicative and when considered with those of other types of measurements seem quite conclusive.

Material and Apparatus

Young's giant axon preparation (Young, 1936) from the Atlantic squid, *Loligo pealii*, was used throughout. The dissection of the hindmost stellar nerve and the teasing of the axon from this nerve have been described (Cole and Curtis, 1939). In the effort to reduce the variability of the results extreme care was taken in the dissection and

particular attention was paid to the elimination of body fluids and to the locating and cutting of the axon branches.

The electrode system was essentially the same as previously used (Cole and Hodgkin, 1939) but with several modifications as shown in Fig. 2. One end of the axon was pulled

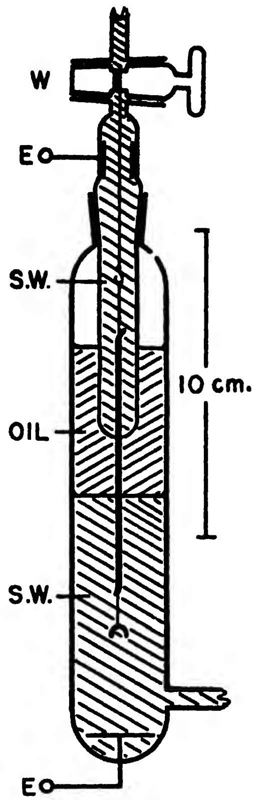

FIG. 2. Longitudinal impedance cell for the squid giant axon. The impedance is measured between the platinized platinum electrodes, E, E, in the sea water surrounding the upper and lower ends of the axon. The vertical position of the axon is varied by the stopcock windlass, W. The interpolar region is in oil and its length is varied by admitting or removing sea water at the lower end of the cell.

up through a small hole in the bottom of a tube filled with sea water and the other end hung through a layer of oil and into sea water in another vessel. Large platinized platinum electrodes made contact with the sea water at each end of the axon. The areas of the electrodes and the high resistance of the preparation made the effect of electrode polarization so small that corrections for it were unnecessary even at 30 cycles. By transferring sea water between the lower vessel and a calibrated burette the interpolar distance was easily varied and accurately measured. The entire axon could be moved up or down in the electrode system by a stopcock windlass which was hooked to the

ligature on the upper end of the axon. A slight tension was kept on the axon by a small platinum weight attached to the lower ligature.

The alternating current Wheatstone bridge and the accessory equipment have been described (Cole and Curtis, 1937), and measurements were made at frequencies from 30 cycles to 200 kc. The measuring potential applied to the axon was as small as possible and the bridge balance was independent of it except when the effect of potential was being investigated. The inductive reactances were measured as in the earlier experiments by connecting a sufficiently large known capacity (0.01 or 0.02 μf) in parallel with the unknown, or axon, arm of the bridge to give it a net capacitative reactance which could be balanced as usual. The axon reactance was then specified in terms of the "negative" parallel capacity.

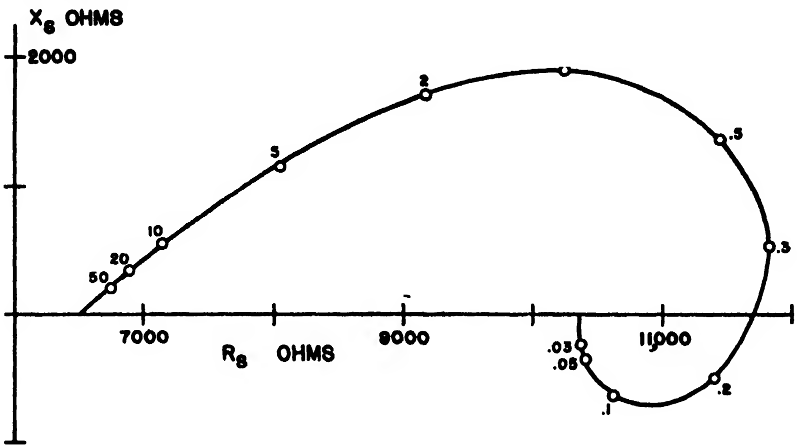

FIG. 3. Longitudinal impedance locus, series resistance, R_s, *vs.* series reactance, X_s, for squid giant axon. Negative, or capacitative, reactances are plotted above the resistance axis. Frequencies indicated are in kilocycles.

The series resistance and reactance, R_s and X_s, at each frequency were calculated from the observed parallel resistance and capacity (positive or "negative") and plotted as the impedance locus (Cole, 1928, 1932).

Measurements and Interpretations

The longitudinal impedance locus of Fig. 3 shows the same phenomena as were observed in the summer of 1938. The behavior at high frequencies was anticipated but the dip below the resistance axis at low frequencies is the unexpected inductive reactance. Although the first axon in the present series of experiments showed these characteristics, it was soon apparent that there was a considerable variability to be contended with. Some axons would show a negative capacity (*i.e.* positive, or inductive, reactance) at frequencies below 200 cycles over their entire length and for all interpolar distances. Other axons would have a low frequency reactance

which was positive for one interpolar distance and negative, or capacitative, for another. Occasionally the change from one to the other took place when the distance was varied as little as a millimeter. More often this reactance would change from positive to negative during the course of an experiment and there were a few cases in which a positive reactance was not found at any time, in any portion. An example of an axon without net inductive reactance is shown in Fig. 4*a*, and it is found that the low frequency portion still indicates the presence of an inductance.

The most obvious variable was that of physiological condition. There were some variations in the subthreshold phenomena but these could not be clearly correlated with the reactance measurements. These axons were

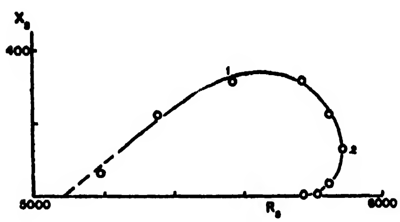

FIG. 4*a*

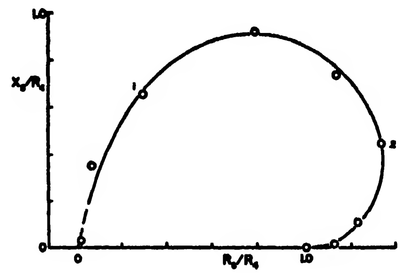

FIG. 4*b*

FIG. 4. (*a*) Longitudinal impedance locus, series resistance, R_s, *vs.* series reactance, X_s, for a squid giant axon without net inductive reactance. (*b*) Membrane impedance locus, calculated from data shown in (*a*) by equation (6). Frequencies indicated are in kilocycles.

all excitable at reasonable thresholds and propagated apparently normal action potentials over their entire lengths and the survival was fair. There was a trend to indicate that the inductive reactance diminished and disappeared as the condition of the axon became poorer. This, however, failed as a generalization when several of the best axons gave capacitative reactances throughout their entire length immediately after removal from the animal. These axons, however, become inductive in the course of an hour or so.

This experiment was obviously so sensitive to some uncontrolled factor or factors that it was essential to eliminate as many uncertainties as possible. The impedance was always independent of the measuring current when this was sufficiently small, but if action potentials and harmonics generated by the non-linearity of the membrane resistance were suppressed or ignored an inductive reactance might apparently become capacitative when the

measuring current was increased. For a number of experiments, the mineral oil was not used and the interpolar stretch was in air. The inductive reactances were of about the same magnitude and as irregular as before, but the survival time of the axons was considerably shortened. Aeration of the oil had little or no effect and fresh sea water did not seem to be essential. Axons with considerable connective tissue gave results comparable to those which were carefully teased. A wide variety of wood, thread, and other "artificial" axons all failed to give an inductive reactance. At one time it was thought that the shape of the upper meniscus was a factor, but the effect of changes of the size of the orifice and the form of the meniscus was finally shown to be negligible as long as the position of the meniscus on the axon was unchanged.

As the search for constant conditions progressed, it became more and more certain that variability and the inductive reactance both lay in the axon and this was supported by the one completely reproducible observation that an inexcitable axon never gave a net inductive reactance at any frequency. Then considerable progress was made by killing one end of the axon with alcohol. With the variability of one electrode region eliminated, it was possible to show that the irregularities of impedance were probably caused by local differences in the axon which had only a slight effect on the subthreshold response, the threshold, or the action potential.

Out of the seventy-four axons investigated, the characteristics of sixty-six may be roughly classified. In nine of these, no negative capacity was found in any part of the axon at any time. Twenty-five axons showed part negative and part positive capacities at low frequencies and in many cases the latter was correlated with a visible injury, while in four cases it was apparently an initial condition. The remaining thirty-two showed negative capacities over their entire length when this was investigated. It is now felt that the initial completely capacitative reactance is the most nearly normal condition and that this is followed by the relatively stable stage in which the low frequency reactance was inductive. Subsequently this net inductive reactance disappeared and the axon became inexcitable.

When the initial capacitative phase was found, its duration was too short and the impedance was too variable for satisfactory impedance measurements to be made over a wide frequency range. As a consequence there is practically no information available about the axon in this condition. At the stage where the low frequency reactance was inductive, the axon impedance would often remain quite constant for several hours and frequency runs could be made. The behavior of the axon shown in Fig. 3 has all the typical characteristics of this stage and does not present any of the extremes encountered.

Localization of Inductive Structure

The next step was to locate the structures responsible for the inductance. As the interpolar distance is increased, the two electrode regions become practically independent and there is then no current flow across the membrane in the central portion of the interpolar region. If the inductance and capacity are both located in the membrane there should be no change in their contribution to the impedance as the interpolar distance is increased beyond five or six times the characteristic length as shown by equation (3). There is, however, current flow along the connective tissue and sea water outside, and through the axoplasm inside the membrane in this central region. If the inductance is located in either of these its contribution would depend upon the electrode separation even when this is large. This obvious and crucial experiment proved to be quite difficult to carry out because it required that the axon be uniform throughout its length and that this uniformity be maintained until the measurements were completed. It would be necessary to make a series of frequency runs with the same interpolar distance in a number of regions along the length of the axon, to prove the uniformity, before similar frequency runs could be made with varying interpolar distance. As has been indicated, sufficiently uniform fibers were not easy to obtain, and it was soon found that bridge measurements over a wide frequency range were too slow for the completion of this program. Furthermore, the capacity sensitivity of the bridge at the lowest frequencies was inadequate for the high resistances encountered at long interpolar distances, and impedance loci at four interpolar distances on each of eight axons did not demonstrate any consistent behavior at long interpolar distances. Since the resistance and reactance at 50 cycles were the most critical single measurements, attention was then centered on them. The variation of the resistance with interpolar distance was quite regular, and although the reactance was often erratic it tended to decrease with distance as often as to increase. On the other hand, for the four axons which gave smooth reactance curves the reactance was independent of distance at sufficiently large electrode separations. The data for the best of these axons are shown in Fig. 5. This may of course be fortuitous, but if the inductance was in the connective tissue or the axoplasm, the membrane capacity would have had to be larger at the longer interpolar distances. Since the membrane capacity at higher frequencies was relatively independent of physiological condition and the present measurements, taken as a whole, indicate no trend, this combination of circumstances seems rather unlikely.

It is found on page 782 that at high frequency the longitudinal current

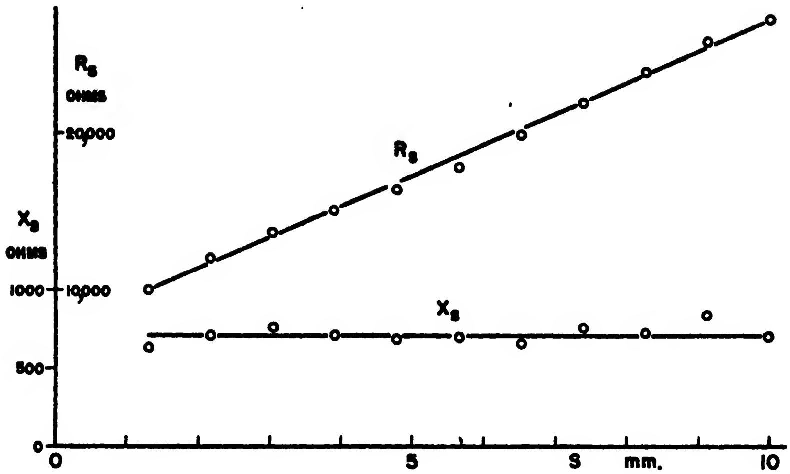

FIG. 5. Series resistance, R_s, and series reactance, X_s, at 50 cycles *vs.* interpolar distance, s, for squid giant axon. The reactances are inductive.

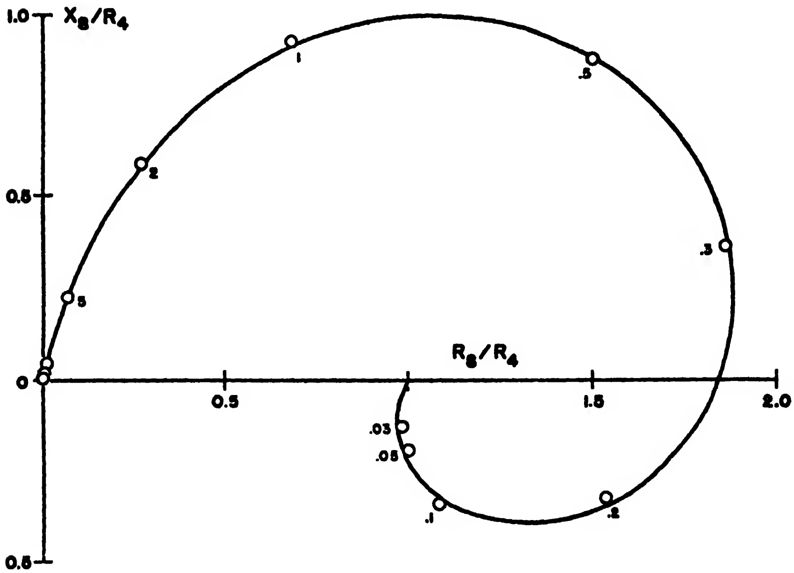

FIG. 6. Membrane impedance locus, calculated from longitudinal impedance data on axon of Fig. 3 by equation (6). Negative, or capacitative, reactances are plotted above the resistance axis. Frequencies indicated are in kilocycles.

would be carried by the axoplasm alone if the connective tissue were inductive, and by the connective tissue alone if the axoplasm were inductive. This has not been found, as is illustrated by Fig. $2d$ (Cole and Hodgkin,

1939) where the current is divided between the axoplasm and the connective tissue in the same ratio at high frequencies as it is with direct current for long interpolar distances, shown by the dotted lines of Fig. 3 in the above paper.

Consequently the experimental data lead us to assume that the axoplasm and the connective tissue are not responsible for the inductance and that it is a characteristic of the membrane.

Membrane Impedance and Equivalent Circuit

When the axoplasm and the connective tissue sheath are pure non-reactive resistances, it is found, equation (6), that the membrane impedance is easily calculated from the measured longitudinal impedance by squaring the frequency dependent part of the latter. In this way, the membrane

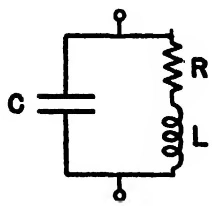

FIG. 7. An approximate equivalent membrane circuit for the squid giant axon, consisting of capacity C, resistance R, and inductance L.

impedances for the data of Figs. 3 and 4*a* have been determined and the loci are given in Figs. 6 and 4*b* respectively.

The impedance characteristics of the membrane are most simply discussed in terms of an equivalent circuit. There are, in general, many possible circuits which can represent a particular set of data (*cf.* Cole 1928, 1937) and the choice of any one should be justified by a theoretical analysis of the structure. At the present time there is not sufficient experimental evidence of the membrane structure to provide the basis for a theory which would uniquely determine a circuit. Consequently the choice will be dictated by utility, convenience, and personal prejudice, and for these reasons the circuit of Fig. 7 is proposed. This equivalent circuit can be seen to have impedances at least approximately those of the axon membranes by a comparison of the theoretical impedance loci for various values of C/L, shown in Fig. 8, with the membrane loci of Figs. 4*b* and 6.

Considering first the membrane impedance in Fig. 6, we see that it is not purely capacitative at high frequency because the locus does not approach the resistance axis at an angle of 90°. The approach at an angle $\phi < 90°$ is, however, to be expected on the basis of an element in the membrane having a dielectric loss and an impedance $z_s = \bar{z}(j\omega)^{-\alpha}$ as has been found in a number

of biological materials. This particular value of $\phi = 75°$ is close to the average of all the axons for which complete frequency data are available and is in good agreement with the values previously obtained (Curtis and Cole, 1938; Cole and Curtis, 1939). Then by considering the high frequency data alone and ignoring the inductance, a time constant of the membrane, $\tau = r_4/|z_m|\omega = 1/|\bar{z}|^2\omega$, can be calculated without a knowledge

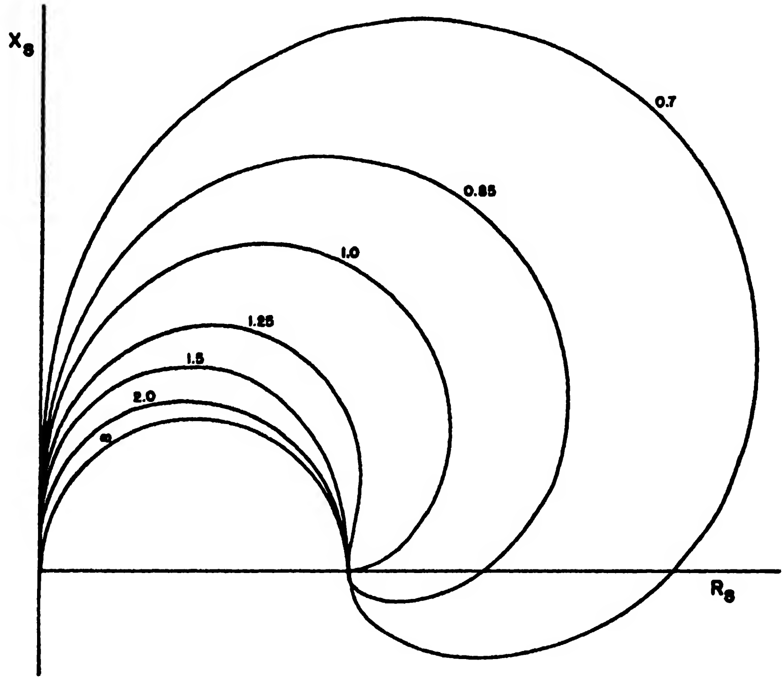

FIG. 8. Theoretical impedance loci, series resistance, R_s, *vs.* series reactance, X_s, for circuits of form shown in Fig. 7. The resistance R is constant throughout and the value of the damping factor, $\eta = R\sqrt{C/L}$, is given for each locus.

of the absolute value of either r_4 or z_m and we find this to be about 0.4 msec. If the membrane capacity is 1 μf/cm². the corresponding membrane resistance is 400 ohm cm²., which is a reasonable value.

The resonant frequency of the membrane at which its series reactance, X_m, is zero, is about 250 cycles in the axon of Figs. 3 and 6, and other axons gave between 150 cycles to 320 cycles for this frequency. The undamped natural frequency of the capacity-inductance combination may now be calculated by equations (11 and 12). For the axon of Figs. 3 and 6, the damping factor $\eta = 0.72$ and $\nu_0 = 360$ cycles, and for the other nine axons for which the complete frequency data are satisfactory, these undamped

natural frequencies lie between 260 cycles and 380 cycles with an average value of 330 cycles. Taking a value of 1.1 $\mu f/cm^2$. for the membrane capacity, this leads to a value for the membrane inductance of 0.21 henry cm^2.

It is possible to go still further with the calculations and obtain a value for the membrane resistance, r_4, by equation (12). For the axon of Figs. 3 and 6 the membrane resistance is 290 ohm cm^2., while the other axons gave resistances from 260 ohm cm^2. to 420 ohm cm^2. with an average of 350 ohm cm^2. These values agree moderately well with those obtained from the high frequencies (p. 780), are definitely lower than the resistances of 400 ohm cm^2. to 1100 ohm cm^2. obtained from direct current measurements (Cole and Hodgkin, 1939), and are higher than the 14 ohm cm^2. to 40 ohm cm^2. obtained from the membrane potential during current flow (Cole and Curtis, 1941).

Theory

Necessity of an Inductance

It seems obvious that if the reactance of any circuit in general, and the axon in particular, is inductive, there must be an inductance in that circuit. There are, however, phenomena characteristic of inductive circuits, such as "over-shoot" and oscillation, which may also be produced in circuits having only resistance and capacity, and no inductance. The interpretation and application of the present results will then be considerably simplified if it can be determined whether or not an inductance is necessary. The impedance, z, of a particular combination of lumped or distributed resistances, inductances, and capacities between two terminals can be calculated by ordinary methods, but at present we cannot assume a specific structure and must use a general analysis. We shall define first a resistance, r, as anything having a potential difference which is proportional, at every instant, to the current flowing through it, $e = ri$. Similarly for an inductance, the potential difference is proportional to the rate of change of current, $e = l\frac{di}{dt}$ and for a capacity the potential difference is proportional to the charge, $e = \frac{1}{c}\int idt$. It then follows that energy is dissipated as heat in a resistance, but is stored in an inductance as kinetic energy and in a capacity as potential energy. These energies will vary as the current and potential difference are changed, but for alternating current, average values may be used conveniently. When an alternating current, I, flows between

the terminals of a known circuit, the average rate of energy dissipation in all the resistances, F, the average kinetic energy in all the inductances, T, and the average potential energy in all the capacities, U, can be calculated. It is not immediately obvious that there should be a connection between these quantities and the impedance, but a relation has been given by Bode (1935) which may be rewritten,

$$Z = R_s + jX_s = 2[F + j\omega(T - U)]/I^2 \tag{1}$$

where $\omega = 2\pi$ times the frequency and $j = \sqrt{-1}$. This equation is particularly useful because it applies to *any* two terminal circuits and because the quantities F, T, U are either positive or zero. If then X_s is positive, $T - U$ and, consequently, T must be greater than zero. Since this is a kinetic energy which is associated only with inductance, it follows that an inductance is necessary. On the other hand if T is zero, X_s must be negative or else zero, but it will be noticed that there may still be an inductance, giving T positive, although X_s is negative.

Location of the Inductance

Since inductance is now necessary, it must be allowed for in the calculation of the longitudinal impedance.

We shall consider the axoplasm and connective tissue to have impedances z_1 and z_2 per unit length of axon, which are perhaps of the form $z = r + j\omega l$, with a membrane impedance z_m per unit length, and obtain (Cole and Curtis, 1936, equation 1; Cole and Hodgkin, 1939, equation 1) for infinite electrodes

$$Z = \frac{z_1 z_2}{z_1 + z_2}\, s + \frac{2z_1^2\lambda}{(z_1 + z_2)[\sqrt{(z_1 + z_2)/z_2} + \coth s/2\lambda]} \tag{2}$$

where s is the electrode separation and $\lambda = \sqrt{z_m/(z_1 + z_2)}$. If s is large and $\coth s/2\lambda$ approaches unity this becomes

$$Z = \frac{z_1 z_2}{z_1 + z_2}\, s + \frac{2z_1^2\lambda}{(z_1 + z_2)[\sqrt{(z_1 + z_2)/z_2} + 1]} \tag{3}$$

where the second term is now independent of the electrode separation and will contribute a constant resistance and reactance. The first term, however, is inductive, if either or both of z_1 and z_2 are inductive, and this reactance will be important at large values of s. At high frequency, the overall impedance should become very high if z_1 and z_2 are both inductive, and should approach $r_1 s$ if z_2 alone is inductive or $r_2 s$ if z_1 alone is inductive. On the basis of experimental evidence (p. 777) we may assume that z_1, z_2 are non-inductive and may be replaced by r_1, r_2, and that the inductance is to be found in the membrane.

Membrane Impedance

Before assuming an equivalent circuit or a location for an inductive element in the membrane, it is helpful to obtain the frequency characteristics or the impedance locus for the membrane alone from the observed longitudinal impedances for the axon as a whole. Equation (2) now becomes,

$$Z = \frac{r_1 r_2}{r_1 + r_2} s + \frac{2r_1^2 \lambda}{(r_1 + r_2)[\sqrt{(r_1 + r_2)/r_2} + \coth s/2\lambda]} \tag{4}$$

where $\lambda = \sqrt{z_m/(r_1 + r_2)}$. It still seems reasonably safe to assume that at high frequencies the current in the membrane is carried primarily by its capacity and that z_m approaches zero. Extrapolating to infinite frequency we have $R_\infty = \dfrac{r_1 r_2}{r_1 + r_2} s$. For direct current, the membrane has been shown to have a resistance, r_4, giving the overall resistance at zero frequency,

$$R_0 = \frac{r_1 r_2}{r_1 + r_2} s + \frac{2r_1^2 \lambda_0}{(r_1 + r_2)[\sqrt{(r_1 + r_2)/r_2} + \coth s/2\lambda_0]}$$

where $\lambda_0 = \sqrt{r_4/(r_1 + r_2)}$. We then find for the frequency dependent part of the longitudinal impedance

$$\bar{Z} = \frac{Z - R_\infty}{R_0 - R_\infty} = \frac{\sqrt{(r_1 + r_2)/r_2} + \coth s/2\lambda_0}{\sqrt{(r_1 + r_2)/r_2} + \coth s/2\lambda} \cdot \sqrt{\frac{z_m}{r_4}}, \tag{5}$$

and for s large,

$$\bar{Z} = \sqrt{z_m/r_4} \quad \text{or} \quad z_m = r_4 \bar{Z}^2 \tag{6}$$

Remembering now that $\bar{Z}$ and z_m are complex quantities, we see immediately that equations (6) are conformal transformations of the simplest kind (Weaver, 1934).

If $\bar{Z} = \bar{R} + j\bar{X}$ and $z_m = r_m + jx_m$, then $r_m + jx_m = r_4(\bar{R}^2 - \bar{X}^2 + j\bar{R}\bar{X})$, from which r_m, x_m may be calculated at each frequency. Expressing both impedances in polar coordinate form

$$\bar{Z} = |\bar{Z}|e^{j\Phi} \quad \text{and} \quad z_m = |z_m|e^{j\phi_m}$$

we have

$$|z_m|e^{j\phi_m} = r_4|\bar{Z}|^2 e^{2j\Phi} \tag{7}$$

and so

$$|z_m| = r_4|\bar{Z}|^2, \qquad \phi_m = 2\Phi$$

The absolute value of the membrane impedance, z_m, is then proportional to the square of the absolute value of $\bar{Z}$, the frequency dependent part of

the longitudinal impedance, and the phase angle ϕ_m of the membrane impedance is twice that of $\bar{Z}$. This calculation is particularly simple to carry out graphically. In the case where the membrane is assumed to be a resistance and capacity in parallel, its impedance is given by $1/z_m = j\omega c_3 + 1/r_4$ and the locus of Fig. 1 *a*. The corresponding longitudinal impedance function, $\bar{Z}$, for such a membrane is then represented by the locus of Fig. 1 *b*. Conversely, where the longitudinal impedance data, such as Figs. 3 and 4 *a*, are available, the membrane impedance is calculated as shown in Figs. 6 and 4 *b*.

Equivalent Membrane Circuit

For many purposes it is convenient to consider the membrane characteristics in terms of an electrical circuit. The impedance of this circuit at any frequency should then be approximately that of a unit area of the membrane. The circuit shown in Fig. 7 has been chosen for reasons to be discussed later. This equivalent membrane circuit contains the capacity element C, which has the impedance $z = \bar{z}(j\omega)^{-\alpha}$ (where $\bar{z}$ and α are constants) characteristic of many dielectrics. With an impedance of this form, relatively simple calculations appear complicated and the meaning becomes obscure to say the least. For this reason, we shall consider here, as a first approximation, the circuit in which C is a pure capacity. The impedance of the circuit is then

$$Z = \frac{(R + j\omega L)/j\omega C}{R + j\omega L + 1/j\omega C} = R\,\frac{1 + j\omega L/R}{1 - \omega^2 LC + j\omega RC} \tag{8}$$

If we now let $Z/R = \bar{Z} = \bar{R} + j\bar{X}$ and $\nu^2 = \omega^2 LC$, $\eta^2 = R^2C/L$ we have

$$\bar{R} + j\bar{X} = \frac{1 + j\nu/\eta}{1 - \nu^2 + j^2\eta} = \frac{1}{(1 - \nu^2)^2 + \nu^2\eta^2} + \frac{j\nu}{\eta}\cdot\frac{1 - \nu^2 - \eta^2}{(1 - \nu^2)^2 + \nu^2\eta^2}. \tag{9}$$

η is the damping factor for the circuit, and ν is proportional to the frequency. At the undamped resonant frequency of the circuit, $\nu \equiv \nu_0 = 1$, and we have

$$\bar{R} + j\bar{X} = 1/\eta^2 + j/\eta \tag{10}$$

Also the reactance X is zero, $\bar{X}(\bar{\nu}) = 0$ at a frequency $\bar{\nu}$, other than zero or infinity, for $\bar{\nu}^2 = 1 - \eta^2$ (11) and $\bar{R}(\bar{\nu}) = 1/\eta^2$ (12) only when $\eta \leqq 1$. Then $\bar{R}(\bar{\nu}) \geqq 1$ and in this case with equation (10) we have a convenient method for determining ν_0 as well as η.

The loci of equation (9) have been plotted in Fig. 8 for several values of η. For $\eta = \infty$, the inductance is negligible and the locus is a semi-circle

determined by the resistance and capacity, and for $\eta = 2.0$, the circuit is critically damped. If $\eta = \sqrt{2}$, the damping is 70.7 per cent of critical or "optimum" which is often preferable to critical damping for recording instruments. The case of $\eta = 1.0$ is anomalous in that the locus approaches the zero frequency resistance along the resistance axis rather than at right angles as it does for all other values of η. As shown by van der Pol (1937) and Bode (1938) the energies stored by the inductances and capacities for direct current are equal in this case.

DISCUSSION

The concept of an inductance in a cell membrane is so foreign to our past experience and so difficult to grasp that we must inquire closely into each of the steps which has led to it before we can resign ourselves to the necessity of accepting and using it. There are observations of potentials and excitabilities which are strongly indicative of an inductive element in the membrane, but the present impedance measurements seem to be the only direct proof of the necessity of such an element. If this proof is not conclusive, evidence of all kinds may at least make an inductance seem reasonable, but if the impedance results alone are adequate proof, the conclusions may then be used as known factors in the interpretation of other phenomena. We shall therefore discuss the present results without reference to other evidence.

The only factors which have not been altered or replaced in the experimental work are the Wheatstone bridge and the squid axon. The bridge and its accessory equipment have been checked so often and in so many ways that it seems highly improbable for an error of 0.01 to 0.02 μf at frequencies of 50 to 200 cycles to appear only when a live axon was in the measuring cell. Three measuring cells with two different types of electrodes have been used and the mineral oil was changed or omitted without effect. It thus seems quite certain that the impedance characteristics are those of the axon. We are then able to prove theoretically that when an inductive reactance is measured there must be an inductance in the axon. This reactance was relatively independent of the external connective tissue on the axon which helps to eliminate it as a factor. The spatial and temporal variability encountered makes one suspect the membrane but does not eliminate the axoplasm. The experiments on the relation between impedance and interpolar distance are not completely satisfactory, but they do not consistently permit either an inductive axoplasm or connective tissue. Furthermore, none of these results eliminate an inductance from the membrane and there are several which can only be reasonably explained

by an inductive structure in the membrane alone. On the other hand, it has not yet been possible to show that an axon with inductive axoplasm or connective tissue can have an impedance locus similar to the loci found experimentally. We are then fairly well justified in assuming, until better evidence is found to the contrary, that the axon has an inductive as well as a capacitative element and that these are both located in the membrane, while the axoplasm and connective tissue are purely resistive.

With the reactive elements located in the membrane, and a sufficient distance between the electrodes, the calculation of the membrane impedance from the longitudinal impedance is found to be unexpectedly simple. Any attempt to interpret these membrane data should be regarded as speculation and accepted only tentatively, but some general features are quite obvious. The behavior at high frequencies is that of a dielectric impedance which has been referred to as the ion-impermeable aspect of the membrane (Curtis and Cole, 1938; Cole and Curtis, 1939). The phase angle and probably the capacity of this impedance agree with the values previously found from transverse measurements. The extrapolated direct current resistance of the membrane has not been carefully measured as a function of the electrode separation, but it is in better than qualitative agreement with other measurements (Cole and Hodgkin, 1939; Cole and Curtis, 1941). As before, we may consider this resistance as a measure of the ion-permeable aspect of the membrane and essentially in parallel with impermeable or capacitative portion. The equivalent circuit previously used for the membrane still seems to be satisfactory at both the very high and the very low frequencies, and we naturally wish to change these characteristics as little as possible when we introduce an inductance into the circuit. The fact that the membrane impedance has a maximum shows that the circuit is anti-resonant with the capacity and inductance in parallel. The direct current resistance must then be in series with the inductance and so we are led to the circuit of Fig. 7. As has been pointed out, many other and more complicated circuits can be found to express the data equally well or better, but we hesitate to consider a more involved situation until it is absolutely necessary for structural or phenomenological reasons. The structural implications of the elements of the present circuit and the application of the circuit as a whole to several physiological phenomena will be considered later (Cole, 1941).

There is adequate reason to believe that the membrane capacity of about 1 $\mu f/cm^2$. is a structural characteristic which is relatively inert and insensitive to physiological and pathological changes. On the other hand, the accumulating evidence emphasizes the importance of the membrane

resistance and supports the growing conviction that this factor either governs or expresses the physiological condition and behavior of the cell. Unfortunately, from the available data it is not yet apparent whether or not the inductive element is a function of physiological condition. Where an undamped natural frequency can be calculated, it is surprisingly constant and is in a frequency range which is significant for a variety of other cells and phenomena. This suggests that the inductance may be as widespread and as constant as the membrane capacity.

There are some objections to the assumed circuit which are quite apparent. It seems fairly certain that as an axon deteriorates the membrane resistance decreases steadily. As this progresses the axon becomes inexcitable and finally the membrane capacity is short-circuited. If this were the only change taking place, the membrane might be initially overdamped, but would become increasingly under-damped and show a larger and larger inductive reactance as the resistance fell. This is not the case, for the inductive reactance vanished before the axon became inexcitable and never reappeared later. The circuit may be modified by the addition of a second variable resistance to include this observation, but the evaluation of an added unknown cannot be undertaken with the present data.

The characteristics shown in Figs. 4 *a* and *b* were found for a number of axons and they are particularly puzzling. From the analysis of the circuit it is found that the impedance locus should only approach the zero frequency resistance along the resistance axis in the case of optimum damping, $\eta = 1.0$, while in all other cases the locus should approach the resistance axis at right angles. It is difficult to blame the circuit for this but it seems unduly optimistic to expect the axon to adjust itself so often to this value of η. Another factor to be borne in mind is that it has been commonly assumed that the resistance in the capacitative arm, other than that representing the dielectric loss, is negligible. This assumption has not been proven and may not be justified.

An application of the present results, and in particular the inference of an inductance, to a normal squid axon and to other materials may be reasonably questioned. The net inductive reactance was not found in the axons which were judged to be the least abnormal, but there is more than a suspicion that the impedance loci of these were similar to Fig. 4 *a*, which also requires an inductance. Until it is possible to work with normal rather than surviving tissue we will not be in a position to do more. On the other hand, the stability of a good axon after it had equilibrated itself was comparable to that achieved in other experiments with this preparation, and we may assume that the process of equilibration and the steady state

were probably similar. If this is true, the longitudinal impedance measurement is at least a very sensitive index of the condition of the axon.

SUMMARY

Longitudinal alternating current impedance measurements have been made on the squid giant axon over the frequency range from 30 cycles per second to 200 kc. per second. Large sea water electrodes were used and the inter-electrode length was immersed in oil. The impedance at high frequency was approximately as predicted theoretically on the basis of the poorly conducting dielectric characteristics of the membrane previously determined. For the large majority of the axons, the impedance reached a maximum at a low frequency and the reactance then vanished at a frequency between 150 and 300 cycles per second. Below this frequency, the reactance was inductive, reaching a maximum and then approaching zero as the frequency was decreased.

The inductive reactance is a property of the axon and requires that it contain an inductive structure. The variation of the impedance with interpolar distance indicates that the inductance is in the membrane. The impedance characteristics of the membrane as calculated from the measured longitudinal impedance of the axon may be expressed by an equivalent membrane circuit containing inductance, capacity, and resistance. For a square centimeter of membrane the capacity of 1 μf with dielectric loss is shunted by the series combination of a resistance of 400 ohms and an inductance of one-fifth henry.

BIBLIOGRAPHY

Bode, H. W., in Guillemin, E. A., Communication networks, New York, John Wiley and Sons, Inc., 1935, **2**, 226; 1938, *Physica*, **5**, 143.

Cole, K. S., 1928, *J. Gen. Physiol.*, **12**, 29; 1932, **15**, 641; 1937, *Tr. Faraday Soc.*, **33**, 966; 1941, *J. Gen. Physiol.*, 1941, **25**, in press.

Cole, K. S., and Curtis, H. J., 1936, Electric impedance of nerve and muscle, in Cold Spring Harbor symposia on quantitative biology, Cold Spring Harbor, Long Island Biological Association, **4**, 73; 1937, *Rev. Scient. Instr.*, **8**, 333; 1939, *J. Gen. Physiol.*, **22**, 649; 1941, **24**, 551.

Cole, K. S., and Hodgkin, A. L., 1939, *J. Gen. Physiol.*, **22**, 671.

Curtis, H. J., and Cole, K. S., 1938, *J. Gen. Physiol.*, **21**, 757.

van der Pol, B., 1937, *Physica*, **4**, 585.

Weaver, W., in Sokolnikoff, I. S., and Sokolnikoff, E. S., Higher mathematics for engineers and physicists, New York, McGraw-Hill, 1934, 441.

Young, J. Z., 1936, Structure of nerve fibers and synapses in some invertebrates, in Cold Spring Harbor symposia on quantitative biology, Cold Spring Harbor, Long Island Biological Association, **4**, 1.

THE INFLUENCE OF ROENTGEN RAYS UPON THE NITROGEN FIXATION BY AZOTOBACTER

By R. M. WHELDEN, E. V. ENZMANN, and C. P. HASKINS

(From the Biological Laboratories, Harvard University, Cambridge, Massachusetts, and the Haskins Laboratories, New York, New York)

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INTRODUCTION

Nitrogen fixation by living organisms must be considered one of the fundamental life processes. The importance of investigations directed towards further elucidation of this little understood process need scarcely be emphasized. During the past four decades, well over a thousand published papers have resulted from the study of just one of these organisms, the free-living aerobic nitrogen-fixing bacterium *Azotobacter*.

Experiments with ionizing radiations have given valuable information in studies on the "sensitive volumes" associated with mutations in *Drosophila* (Haskins and Enzmann, 1936; Enzmann and Haskins, 1938), and on the mechanism of photosynthesis in *Chlorella pyrenoidosa* (Arnold, 1933); and experiments with a similar purpose have been made with low voltage cathode rays on *Aspergillus* (Whelden and Haskins, 1938; Buchwald and Whelden, 1939; Whelden *et al.*, 1940). Work employing radiations in the study of the mechanism of nitrogen fixation by *Azotobacter* is very meager.

The influence of visible light upon the rate of nitrogen fixation has been noted by several workers, but no comprehensive study has been made. It has been stated that nitrogen fixation takes place in light as well as in darkness. Kayser (1920) tested the influence of visible light upon N-fixation by using differently colored glass containers as culture vessels. He reported that nitrogen fixation was small in violet-colored vessels and large in brown ones. Mannite as energy source was completely used up in 3 months at room temperature in brown, green, and black containers. The same author (Kayser, 1921) tested the influence of uranium salts (acetates and nitrates) upon N-fixation and found that the utilization of mannite as well as nitrogen fixation was increased thereby. With glucose as energy source the effect was even more pronounced. The addition of powdered radioactive salt to culture media (Kayser and Delaval, 1924) increased nitrogen fixation by as much as 75 per cent. Stoklasa (1920) experimented with potassium which emits β and γ rays, and agreed with others who had reported it to stimulate various life processes such as photosynthesis and embryonic development. He found that radium emanations at amounts of 80 to 150 ME (*Millieinheiten*) were not harmful to nitrogen fixation, and indeed even stimulated it.

The present investigations attempt to answer the following questions:

1. What functional relationship can be obtained between nitrogen fixation and graded x-ray doses?

2. Does exposure to x-rays affect respiration and nitrogen fixation in the same way and to the same degree?

Material and Methods

The three species of *Azotobacter* used in the present work: *Azotobacter chroococcum*, *A. agilis*, and *A. vinelandii* were originally obtained through the kindness of Dr. Dean Burk and have been cultured in our laboratory on agar slants from which inoculations were made to liquid cultures as required. The organisms were grown in liquid culture in 250 cc. Erlenmeyer flasks closed with perforated rubber stoppers holding the glass and rubber aerating systems. The rate of air flow could be regulated by a set screw on each valve. The rate of bubbling of air through the cultures, the temperature of the incubator, the composition of the medium, and the handling of the cultures were in accordance with the methods described by Burk (1930). Burk's method of culturing in gas wash-bottles was also used at first, but was abandoned since it was more difficult to handle and did not give any better results than the method here described.

Azotobacter cultures were incubated up to 24 hours, during which time the cell count rose to 30,000–150,000 cells per cubic millimeter, depending mainly on the rate of aeration.

All counts were made with a Neubauer hemocytometer after diluting the culture to an approximate cell count of 20,000–30,000 cells per cubic millimeter. A drop of acetic acid added to 20 cc. of culture medium shortly before counting stops all motility of the cells and facilitates counting by increasing their refractive index.

After removing the cultures from the aerating system they were tested for pH with brom thymol blue solution; for sugar content with Nessler solution;[1] for freedom from contamination, by microscopic examination; for cell density by cell count or by centrifuging. Suitable samples of cultures were well shaken to insure even cell distribution and divided into two portions of 30 cc. each, one of which was x-rayed while the other was kept as a control. The x-raying was done in Harvard jars at 35 cm. target distance with a Coolidge x-ray machine, delivering 330 Roentgen units per minute at 168 kv. peak voltage and 10 milliamperes. No filters were used. The x-ray doses employed were 500 r., 1000 r., 3000 r., 3300 r., 5000 r., 10,000 r., and 20,000 r. Two controls were run simultaneously with each set of experiments: one unrayed culture and a H_2O control.

Nitrogen fixation was measured by the method used and described by Burk in his experiments demonstrating N-fixation by manometric methods (1930*a*).[2] Since previous workers have stated that the efficiency of N-fixation reaches a maximum at high partial pressures of gaseous nitrogen (Meyerhof and Burk, 1928; Burk, 1930*b*), we used a prepared gas mixture, containing about 1 per cent O_2.

In handling the Warburg manometers we generally followed the method employed by Burk to demonstrate nitrogen fixation gasometrically, except for minor modifications.

[1] By a method described by Burk, 1930 *a*.

[2] Pp. 1178–1179 for technical details.

The gas mixture we used contains less oxygen than that recommended for maximal efficiency of nitrogen fixation by Burk, but has the advantage that the geometry of the apparatus permits a complete gas analysis.

The success of the experiment depended mainly on the accuracy of measuring small quantities of gas volumes. The greatest single source of error was the determination of the manometer reading corresponding to the starting point. This depends on temperature adjustments, degassing of liquids, and the gas consumption by the organisms. The first two factors tend to raise the manometer level, the last reduces it. Corrections are applied the following way. Temperature equilibration produces manometer reading changes which follow Newton's law and which under our experimental conditions are virtually completed in 15 minutes. The degassing of the liquids in the Warburg vessels, or more correctly the establishment of new partial pressures of gases in the manometers and corresponding changes in the proportion of dissolved gases, requires 30–60 minutes and proceeds nearly linearly with time in the final stage. The decrease in gas space due to respiration and nitrogen fixation takes place as soon as the vessels are filled and the stopcocks are closed. The corrections are determined by observations on control vessels containing H_2O instead of cultures. The importance of applying such corrections is shown by the fact that the establishment of new gas equilibria produces an average change in gas volume equal to about 20 per cent of the volume of the nitrogen removed during fixation.

The influence of the hydrostatic pressure of the manometer fluid upon the measurement of gas volumes was eliminated largely by adjusting the manometer levels so that they would be as far above the zero mark (= 150 mm.) at the start of the experiment, as they would be below the zero mark at the end.

Most of the experiments were terminated about 4 hours after x-raying the material, in order to avoid the possible recovery due to new cell generations of *Azotobacter*. Furthermore, carrying the experiments to completion, *i.e.* until the cells had consumed all available oxygen, was found to add little to the results since about 65 per cent of the oxygen is consumed during the first 3 hours and the rate of oxygen consumption as well as the efficiency of nitrogen fixation declines considerably at very low oxygen pressures.

RESULTS

A. Respiration

The experiments summarized below were done under uniform standard conditions defined by:

Age of culture	20–24 hrs,
Cell count	30–200 thousand cells/c.mm.,
Cell picture	free of contaminants,
pH	7.0–7.3,
Bath temperature	28.2 ± 0.02°C.,
Shaker speed	140 cycles/min.,
Duration of run	3 hrs.

At the end of each run the remaining oxygen was removed with alkaline pyrogallol, by mixing the culture with 0.3 cc. of concentrated pyrogallic

acid and 0.3 cc. saturated solution of KOH. The rate of respiration of a normal unrayed culture of *Azotobacter* compared with that of cultures which had received a dose of 3300 r. is shown by Fig. 1. The vessels contained atmospheric air.

All curves made under the same conditions show a slight initial rise in the rate of respiration followed by a period of almost constant rate. When the same experiment is repeated under an atmosphere of 99 per cent N_2 and 1 per cent O_2 the initial rise in the rate of respiration is extremely brief and is quickly followed by a decline in respiration rate (Fig. 2). Very little difference has been found between x-rayed and untreated cells in the rate of oxygen consumption per cell per hour, within the radiation dosages

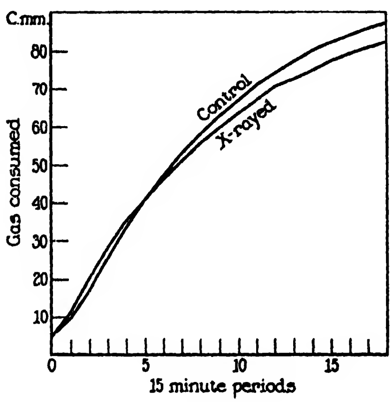

FIG. 1

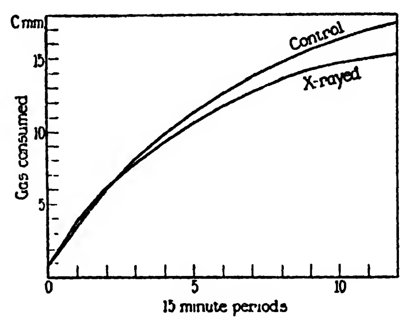

FIG. 2

used ($0.278\mu^3$ O_2/cell/hour for unrayed controls; $0.280\mu^3$ O_2/cell/hour for x-rayed cells are average amounts). It may be concluded that respiration is not markedly affected by x-raying the cells within the dosage range represented by the present experiments. A slight stimulation of respiration was often observed in x-rayed cultures, which is shown in the early periods in Figs. 1 and 2. It is noted that the final rate of oxygen consumption of x-rayed cells is slightly lower than that of the unrayed controls, although it was nearly identical at the start of the experiment. This is explained by the slower rate of cell division of x-rayed cells. Actual counts of x-rayed samples and control samples give figures which are commensurate with the measured differences in the rate of respiration.

B. Nitrogen Fixation

In contrast to respiration, nitrogen fixation is markedly affected by x-irradiation. This is shown in Table I. Column I indicates the treatment

of the culture, column II the total fall of the manometer level due to removal of oxygen as well as nitrogen by respiration and nitrogen fixation, as well as by KOH and pyrogallic acid. Column III shows the amount of oxygen and nitrogen removed by respiration and nitrogen fixation alone, column IV the amount of nitrogen fixed, and column V the amount of oxygen used up in respiration while fixation went on. All volumes are in cubic millimeters at normal temperature and pressure. The contents of the table are visualized easier by reference to the diagram (Fig. 3) drawn to scale, which also illustrates the method of calculating the efficiency of nitrogen fixation.

TABLE I

Showing the Influence of X-Raying with Various Doses upon Respiration, Nitrogen Fixation, and Efficiency of Nitrogen Fixation by Azotobacter

I	II	III	IV	V	VI	VII
Treatment applied	Total fall, $O_2 + N_2$ removed	$O_2 + N_2$ removed by respiration	N_2 fixed	O_2 used up during fixation	Efficiency of fixation	No. of experiments performed
					per cent	
H_2O controls	163.3	0.0	0.0	0.0	—	146
0 r. controls	172.8 ± 0.40	117.3	9.5	107.8	8.75	211
500 r.	172.4 ± 0.41	118.2	9.4	108.8	8.70	74
1000 r.	171.3 ± 0.82	112.9	8.3	104.6	8.0	71
3000 r.	169.9 ± 0.34	111.7	6.9	104.8	6.5	61
5000 r.	167.5 ± 0.42	103.1	4.5	108.6	4.6	76
10000 r.	164.6	108.5	0.9	107.6	0.83	23

Beside each horizontal bar is indicated the nature of the experiment and the bar itself represents the fall of the manometer level during the experiments. The length of the bar marked H_2O indicates the number of cubic millimeters of O_2 and CO_2 removed by KOH and pyrogallic acid. This amount would have been removed from all vessels had they contained H_2O instead of living *Azotobacter*. The excess drop in culture vessels over that in H_2O containing vessels measures therefore the amount of nitrogen removed by *Azotobacter* from the gas mixture, or the amount of nitrogen fixed. Column IV of Table I as well as Fig. 3 show that unrayed cultures fix the largest amount of nitrogen, and that the amount of N_2 fixed decreases regularly with increasing x-ray dose.

The initial experiments (500 r.–5000 r.) showed that nitrogen fixation decreased linearly with increasing x-ray dose. One could therefore by extrapolation determine the x-ray dose which would abolish N-fixation completely. Fig. 4 shows that this dose should be about 11,000 r. Subsequent

determinations with very high x-ray doses (10,000 r., 20,000 r.) showed that the 100 per cent effective dose is higher than expected and that the decrease of N-fixation as a function of x-ray dose is not strictly linear.

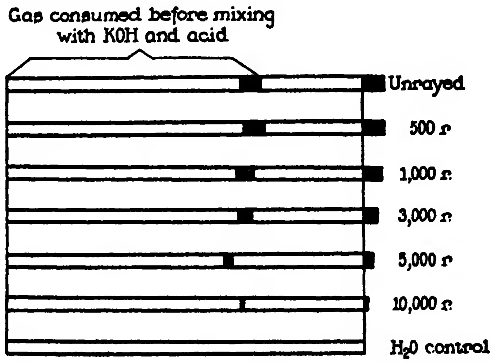

FIG. 3

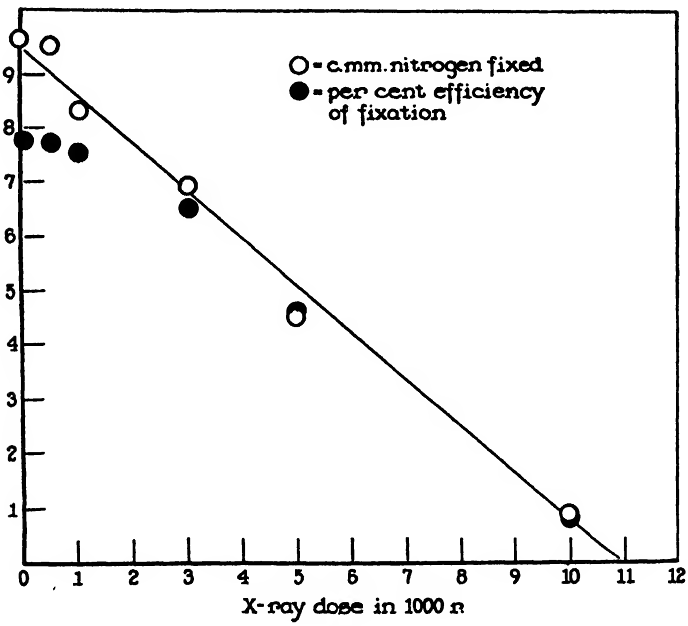

FIG. 4

C. Efficiency

The efficiency of nitrogen fixation is defined as the amount of nitrogen fixed divided by the amount of oxygen used up during the time when fixation took place.

Reference to the diagram (Fig. 3) shows that the true amount of O_2 used in respiration can be found by subtracting the amount of nitrogen fixed (striped bars) from the total decrease in gas space (indicated by bracket and legend), before KOH and pyrogallic acid were mixed with the cultures and the germs were killed. Table I (column VI) shows that the efficiency of fixation also decreases regularly with an increase of the x-ray dose.

It should be noted that several experiments were carried on until practically all oxygen was removed by respiration, and spilling of KOH plus pyrogallic acid produced no further fall. These control experiments agreed substantially with the ones detailed in the table, the total amounts of nitrogen fixed being but slightly higher.

DISCUSSION

It is generally thought that respiration as well as nitrogen fixation are chain reactions governed by enzyme systems. The system involved in N-fixation by *Azotobacter* has been discussed by Burk (1934). Several other schemes have been put forward, most of which involve the assumption that the energy derived from the respiration is used to drive the second mechanism namely nitrogen fixation. This may be represented as follows:

$$\text{Respiration: sugar} + O_2 \rightarrow A \rightarrow B \rightarrow CO_2 + H_2O.$$
$$\text{N-fixation: } N + H_2O \rightarrow P \rightarrow Q \rightarrow \text{fixed N (protein).}$$

The present experiments indicate that the respiratory chain of events as a whole is not affected to any great extent by x-rays of the doses used. Nitrogen fixation however is affected and decreases in a regular fashion with increasing x-ray dose. It can therefore be stated with some assurance, that the two processes, respiration and N-fixation, can be dissociated to an extent depending on the x-ray dose. Such a dissociation of the two processes is not entirely new and may take place spontaneously; a considerable number of students have reported a sudden loss of the power of *Azotobacter* to fix nitrogen. The dissociation can also be brought about by offering to *Azotobacter* certain energy foods which support respiration more or less completely but on which the organism is unable to grow. (We shall report on these experiments in a later communication.)

Arnold (1933) reported a similar dissociation of respiration and photosynthesis, when *Chlorella* was irradiated with ultraviolet light.

SUMMARY

The influence of graded x-ray doses upon nitrogen fixation and respiration by *Azotobacter* was studied by means of the Warburg method. It was found that nitrogen fixation decreases approximately linearly with in-

creasing x-ray doses. Respiration in contrast is affected only indirectly through some inhibition of cell multiplication. Small doses of x-ray often produce a slight and transient increase in the rate of oxygen uptake.

LITERATURE CITED

Arnold, W., 1933, The effect of ultraviolet light on photosynthesis, *J. Gen. Physiol.*, **17**, 135.

Buchwald, C. E., and Whelden, R. M., 1939, Stimulation of growth in *Aspergillus niger* under exposure to low velocity cathode rays, *Am. J. Bot.*, **26**, 778.

Burk, D., 1930 *a*, The influence of nitrogen gas upon the organic catalysis of nitrogen fixation by *Azotobacter*, *J. Physic. Chem.*, **34**, 1195.

Burk, D., 1930 *b*, The influence of oxygen gas upon the organic catalysis of nitrogen fixation by *Azotobacter*, *J. Physic. Chem.*, **34**, 1174.

Burk, D., 1934, Azotase and nitrogenase in *Azotobacter*, *Ergebn. Enzymforschung*, **3**, 23.

Burk, D., and Lineweaver, H., 1930, The influence of fixed nitrogen on *Azotobacter*, *J. Bact.*, **19**, 389.

Enzmann, E. V., and Haskins, C. P., 1938, A determination of the cell sensitive volume associated with the white-eye mutation in x-rayed *Drosophila*. III, *Proc. Nat. Acad. Sc.*, **24**, 136.

Haskins, C. P., and Enzmann, E. V., 1936, A determination of the cell sensitive volume associated with the white-eye mutation in x-rayed *Drosophila*. II, *Proc. Nat. Acad. Sc.*, **22**, 397.

Kayser, E., 1920, Influence de radiations lumineuses sur un fixateur d'azote, *Compt. rend. Acad. sc.*, **174**, 969.

Kayser, E., 1921, Influence des sels d'urane sur le fixateur d'azote, *Compt. rend. Acad. sc.*, **172**, 1133.

Kayser, E., and Delaval, H., 1924, Radioactivité et fixateurs d'azote, *Compt. rend. Acad. sc.*, **179**, 110.

Lineweaver, H., 1932, Characteristics of oxidation by *Azotobacter*, *J. Biol. Chem.*, **99**, 575.

Meyerhof, O., and Burk, D., 1928, Über die Fixation des Luftstickstoffes durch Azotobacter, *Z. phys. Chem.*, *Abt. A*, **139**, 117.

Meyerhof, O., and Schulz, W., 1932, Über die Abhängigkeit des Azotobacter vom Sauerstoffdruck, *Biochem. Z.*, *Berlin*, **250**, 35.

Stoklasa, J., 1920, Über die Radioaktivität des Kaliums und ihre Bedeutung in der chlorophyllosen und chlorophyllhaltigen Zelle. II. Der Mechanismus der physiologischen Wirkung der Radiumemanation und der Radioaktivität des Kaliums auf die biochemischen Vorgänge bei dem Wachstumsprozess der Pflanzen, *Centr. Bact.*, **54**, 72.

von Szent-Györgyi, A., 1939, On oxidation, fermentation, vitamins, health and disease, Baltimore, The Williams & Wilkins Company.

Whelden, R. M., Buchwald, C. E., Cooper, F. S., and Haskins, C. P., 1940, Electron bombardment of biological materials. II. The rate of death of fungus spores bombarded in vacuum with cathode ray beams from 4 kv.–15 kv., *J. Gen. Physiol.*, **23**, 390.

Whelden, R. M., and Haskins, C. P., 1938, Note on an unusual transmitted lethal effect in x-rayed Sorghum, *Bot. Gaz.*, **99**, 874.

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